<table>
<thead>
<tr>
<th>Title</th>
<th>Effect of scavenging circulating reactive carbonyls by oral pyridoxamine in uremic rats on peritoneal dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>森 良孝</td>
</tr>
<tr>
<td>Citation</td>
<td>Nagasaki University (長崎大学) 博士 医学 (2016-12-07)</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2016-12-07</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/36975">http://hdl.handle.net/10069/36975</a></td>
</tr>
<tr>
<td>Rights</td>
<td>© 2016 International Society for Apheresis, Japanese Society for Apheresis, and Japanese Society for Dialysis Therapy; This is the accepted version of the following article: Therapeutic Apheresis and Dialysis, 20(6), pp.645-654; 2016, which has been published in final form at <a href="http://dx.doi.org/10.1111/1744-9987.12446">http://dx.doi.org/10.1111/1744-9987.12446</a></td>
</tr>
</tbody>
</table>
Effect of scavenging circulating reactive carbonyls by oral pyridoxamine in uremic rats on peritoneal dialysis

Yoshitaka Mori¹,², Takatoshi Kakuta¹,³, Takayo Miyakogawa¹, Susumu Takekoshi⁴, Hiroko Yuzawa⁵, Hiroyuki Kobayashi⁶, Atsushi Kawakami², Toshio Miyata⁷, Masafumi Fukagawa¹

1. Department of Nephrology, Endocrinology and Metabolism, Tokai University School of Medicine, Isehara, Japan.
2. Unit of Translational Medicine, Department of Endocrinology and Metabolism, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.
3. Department of Nephrology, Endocrinology and Metabolism, Tokai University Hachioji Hospital, Hachioji, Tokyo, Japan.
4. Division of Basic Molecular Medicine, Tokai University School of Medicine, Isehara, Japan.
5. Department of Neurology, Tokai University School of Medicine, Isehara, Japan;
6. Department of Clinical Pharmacology, Tokai University School of Medicine, Isehara, Japan.
7. United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan.

Corresponding author:
Takatoshi Kakuta, M.D., Ph.D.
Division of Nephrology, Endocrinology and Metabolism
Department of Medicine
Tokai University Hachioji Hospital
1838 Ichikawa-cho, Hachioji
Tokyo 192-0032
Japan
Phone: +81-
Fax: +81-
E-mail: kt99955@tsu-tokai.ac.jp

Running title: Oral pyridoxamine during PD
ABSTRACT:
Pyridoxamine, a reactive carbonyl (RCO) scavenger, can ameliorate peritoneal deterioration in uremic peritoneal dialysis (PD) rats when given via dialysate. We examined the effects of scavenging circulating RCOs by oral pyridoxamine. Rats underwent nephrectomy and 3 weeks of twice daily PD either alone or with once daily oral pyridoxamine. PD solution was supplemented with methylglyoxal, a major glucose-derived RCO, to quench intraperitoneal pyridoxamine. Oral pyridoxamine achieved comparable blood and dialysate pyridoxamine concentrations, suppressed pentosidine accumulation in the blood but not in the mesenterium or dialysate, and reduced the increases in small solute transport and mesenteric vessel densities, with no effects on submesothelial matrix layer thickening or serum creatinine. Thus, reducing circulating RCOs by giving oral pyridoxamine with PD provides limited peritoneal protection. However, orally given pyridoxamine efficiently reaches the peritoneal cavity and would eliminate intraperitoneal RCOs. Oral pyridoxamine is more clinically favorable and may be as protective as intraperitoneal administration.

KEY WORDS: Carbonyl stress, glucose degradation products, mesothelium, neovascularization, pentosidine, peritoneum.
Peritoneal dialysis (PD) is a modality of renal replacement therapy, with efficacy comparable to hemodialysis. In substantial numbers of patients, however, PD has to be discontinued primarily due to peritoneal membrane dysfunction and ultrafiltration failure. Peritoneal small solute transport progressively increases with time on PD, which enhances the dissipation of osmotic gradient, and reduces ultrafiltration capacity (1, 2). Histologically, submesothelial fibrosis and thickening, peritoneal mesothelium denudation, and submesothelial neovascularization occur gradually and steadily during long-term PD (3-6). The magnitude of peritoneal solute transport is determined by the peritoneal vascular surface area, which is dependent upon the number of perfused peritoneal capillaries (7, 8). An increase in dialysis-to-plasma ratio of creatinine (D/P-Cre), an index of peritoneal membrane function, parallels an increase in the peritoneal vascular surface area.

Gathered evidence has cast a new light on the molecular mechanisms of the decline in peritoneal membrane function (9). Biochemical alterations in the peritoneum inherent to uremia are, at least in part, accounted for by "peritoneal carbonyl stress", an overload of reactive carbonyl compounds (RCOs) originating from PD fluid and uremic circulation (9-12). Heat-sterilization of glucose-containing PD fluid generates such glucose degradation products as methylglyoxal, glyoxal, and 3-deoxyglucosone, all of which are RCOs (13). RCOs generated in uremic circulation diffuse into the peritoneal cavity due to osmotic gradient (11). RCOs are capable of forming adducts with proteins by the carbonyl-amine chemistry, leading to the formation of advanced glycation end products (AGEs) and advanced lipoxidation end products (10, 11). AGEs bind to cell via receptors such as the receptor of AGE (RAGE) and induce production of cytokines, growth factors, and reactive oxygen species (14-16). Subsequently, inflammatory cells and myofibroblasts infiltrate and proliferate, leading to submesothelial fibrosis and chronic inflammation (15, 16). Neovascularization also occurs via overexpression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (14, 17-19).

Aminoguanidine and some B6 vitamers inhibit AGE formation (20-22). Of those, pyridoxamine (PM) acts by entrapping RCOs (20, 21, 23). We have previously reported that PM, given via dialysate to uremic rats on PD, ameliorated functional and morphological alterations of the peritoneal membrane (24). We showed that PM suppressed the accumulation of pentosidine, a representative AGE, VEGF mRNA overexpression, the increase in capillary density of the mesenterium, and the elevation in small solute transport. This study raised one question as to whether the benefits of intraperitoneal PM were solely due to its action in the peritoneal cavity or also to its action in the circulation because it is unknown whether PM can cross the peritoneum.
into the circulation while circulating RCOs are known to contribute to "peritoneal carbonyl stress". If PM can cross the peritoneum, PM can be orally applied to eliminate RCOs in both the circulation and peritoneal cavity.

The present study examined whether scavenging RCOs in the circulation by oral PM provides benefits in uremic rats on PD. To this end, we loaded PD fluid with a high concentration of methylglyoxal (MG), a major reactive carbonyl product of glucose degradation, to quench PM if it crossed the peritoneum. Our results showed that scavenging RCOs in the circulation decelerated morphological and functional deterioration of the peritoneum in uremic rats on PD, although adequate PD efficacy was not maintained. The results also showed that orally given PM could cross the peritoneum with significant efficiency. Oral PM has a potential to protect the peritoneal membrane in long-term PD patients.
MATERIALS AND METHODS

Study design

The experimental protocol is illustrated in Figure 1. The experimental protocol and procedures were approved by the Animal Experiment Committee of Tokai University School of Medicine.

Seven-week-old male Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan) were randomized to three groups: controls with sham operation alone (Ctrl; N=7), uremic rats undergoing PD (NxPD; N=8), and uremic rats undergoing PD with oral PM (NxPM; N=7). NxPD and NxPM underwent 5/6 nephrectomy as described previously (24). Two weeks later, Rats-o-ports, each with a 13.5-cm catheter (Access Technologies, Skokie, IL, USA), were subcutaneously implanted in the neck of the nephrectomized animals, and the catheter tip was inserted into the abdominal cavity via the abdominal wall. On surgery, animals were anesthetized with isoflurane, and 0.2 mg of gentamycin was given intramuscularly. Animals were fed a standard rodent diet (CE2: CLEA Japan, Inc., Tokyo, Japan) and had free access to food and water.

One week after the implantation, twice daily peritoneal dialysis with 30 mL of a 3.86% glucose dialysate (Dianeal PD-2 4.25%, Baxter Health Care Corp., Round Lake, IL, USA) was initiated and continued for 3 weeks. Dwell time was 1 hour. In order to quench the activity of PM in the peritoneal cavity if PM crossed the peritoneum, PD solution was supplemented with 10 mmol/L of MG. This concentration of MG can induce full-scale PD-associated morphological alterations of the peritoneum in rats by 21 days of daily dialysis (25-27). Gentamycin was added to the dialysate (5 mg per 2000 mL). The dialysate was infused via the Rat-o-port through a syringe filter (Sartorius Japan, Tokyo, Japan).

PM (50 mg per rat in 2 mL of distilled water) was given by gavage to the NxPM animals once daily one hour prior to dialysate infusion for 3 weeks. An equal amount of distilled water was given to the Ctrl and NxPD animals.

Small solute transport

Peritoneal equilibration test (60 minutes) was performed on the day of sacrifice as described (24). One hour after gavage of PM or distilled water, 30 mL of dialysate was infused. One hour later, approximately 2 mL of the dialysate was collected from the abdominal cavity with a 23G needle syringe directly via the abdominal wall. Simultaneously, 1.5 mL of blood was collected from the caudal vein. Creatinine and urea concentrations of the plasma and the dialysate were measured and used to calculate 60-minute dialysate-to-plasma ratio for creatinine (D/P-Cre) and urea (D/P-UN), respectively.
Blood, dialysate effluent and tissue sampling

After the equilibration test, rats were killed by exsanguination via cardiac puncture under anesthesia. Blood was sampled for biochemical measurements. Dialysate effluents were collected for the measurements of vitamin B6 and pentosidine. A portion of the parietal peritoneum, about 2×2 cm in size, was collected along with the underlying muscle layers from the midline of the abdomen. The mesenterium was harvested by cutting it off from the retroperitoneums and intestinal tracts.

Creatinine, urea, total cholesterol, and triglyceride concentrations of the plasma and dialysate effluent were measured using an autoanalyzer (Hitachi Model 736-60: Hitachi Electronics Co., Ltd., Tokyo, Japan).

Pyridoxamine measurement

Vitamin B6 and vitamer concentrations of the serum and dialysate effluent were determined as previously described (28). Blood and dialysate effluent were protected from light, stored on ice, and analyzed at SRL Inc (Tokyo, Japan). The concentrations of PM, pyridoxine and pyridoxal were determined with high-performance liquid chromatography (HPLC) after their phosphorylated forms were hydrolyzed. Therefore, pridoxamine-5-phosphate, pyridoxine-5-phosphate, pyridoxal-5-phosphate were measured in their corresponding free forms.

Pentosidine measurement

Accumulation of AGEs was assessed in the plasma, mesenterium and dialysate effluent by determining pentosidine, a representative AGE, by the HPLC assay as previously described (24, 28). Approximately 100 mg of peritoneal tissues were homogenized with 1.5 mL of chloroform/methanol (2:1) and subsequently with 1.0 mL of methanol to remove lipids. The homogenized tissue was dried in vacuo and hydrolyzed by 500 μL of 6 N HCl for 24 hours at 110°C. Acid hydrolysates were dried in vacuo, reconstituted with 500 μL of distilled water, filtered through a 0.5 μm-pore filter, and diluted five times with distilled water. The diluted sample was injected into an HPLC system (Prominence HPLC; Shimadzu, Tokyo, Japan) and separated on a C18 reverse-phase column (5 μm, 4.6×250 mm; Nomura Chemical Co., Aichi, Japan). The eluate was monitored with an excitation/emission wave length of 335/385 nm. Synthetic pentosidine was used to obtain a standard curve.

Plasma and dialysate effluent were deproteinized by addition of 5% trichloroacetic acid, dried in vacuo, and acid hydrolyzed by 100 μL of 6 N HCl for 16 hours at 110°C. Acid hydrolysates were dried in vacuo, reconstituted with 400 μL of
distilled water, filtered through a 0.5-μm pore filter, diluted thrice with distilled water and analyzed on the HPLC system described above.

**Histology and immunohistochemistry**

Tissue samples were fixed in 10% formaldehyde, paraffin-processed and embedded, and 5-μm sections were prepared. Parietal peritoneal wall samples were morphologically analyzed with hematoxylin and eosin stain (HE) staining. The thickness of submesothelial matrix layer (SML), the connective tissue between the mesothelium and the muscle layer, was measured in five randomly chosen fields in each tissue section, and the average thickness (μm) was recorded. We confirmed the absence of peritonitis (e.g., granulocytes infiltration) by histologic analysis of peritoneal tissue specimens.

Formalin-fixed paraffin-embedded sections were used to detect VEGF-positive cells using anti-VEGF antibody (1:10; Abcam, Cambridge, MA, USA). Bound antibodies were visualized with 0.2% 3,3-diaminobenzidine tetrahydrochloride (DAB). Positive cells were counted in three randomly chosen fields, and the average value was expressed as the density of positive cells per SML area (mm²).

Blood vessels and lymphatic vessels were detected by double-staining with anti-aminopeptidase P antibody (1:100; Bender MedSystems, Vienna, Austria) as a marker of blood vessels and anti-podoplanin antibody (1:100; AngioBio, Del Mar, CA, USA) as a marker of lymphatic vessels. DAB and nitro-blue tetrazolium chloride/5-bromo-4-chloro-3’-indolylphophatase p-toluidine salt (Nichirei, Tokyo, Japan) were used as chromogenic substrates to visualize immunolabeling of aminopeptidase P and podoplanin, respectively. Vessels were counted in three randomly chosen fields, and the average value was expressed as the density of vessels in the mesenterium (per mm²).

Anti-aminopeptidase P staining requires heat-induced epitope retrieval. However, 15 minutes of autoclaving at 121°C damaged SML, and vessels could not be assessed. For this reason, we used HE-stained sections to assess the number of vessels in SML of the parietal mesothelium. The average number of blood vessels in three randomly selected fields was determined and expressed as the density of blood vessels in SML (per mm²).

**Statistical analysis**

The values are expressed as the mean ± SD. Statistical analysis was performed using analysis of variance (ANOVA) and, where appropriate, the Tukey test was used as a multiple comparison t-test. Correlation analyses were performed using Pearson’s correlation test. The significance level was set at P<0.05.
RESULTS

PM concentrations in the circulation and dialysate effluent
PM concentrations in the blood and dialysate effluent are shown in Figure 2. Blood was sampled 2 hours after oral PM. The dialysate was infused 1 hour after oral PM, and the effluent was recovered after 1 hour of dwelling. In NxPM, PM concentration was 21,363.3±3,856.1 ng/mL in the blood (vs. 1.3±0.1 and 1.0±0.4 ng/mL in Ctrl and NxPD, respectively; \(P<0.001\)) and 20,968.3±2671.4 ng/mL in the dialysate effluent (vs. 0.5±0.1 and 0.2±0.0 ng/mL in Ctrl and NxPD, respectively; \(P<0.001\)).

Pentosidine content
The pentosidine content was measured in the mesenterium (Figure 3A), dialysate effluent (Figure 3B) and blood (Figure 3C). NxPD showed significant increases in all sample types (\(P<0.001\) vs. Ctrl). Oral PM significantly suppressed the increase in the blood (\(P=0.018\) vs. NxPD), but not in the mesenterium or dialysate effluent.

Blood biochemistry and body weight
NxPD showed significant increases in creatinine (\(P<0.001\)), urea (\(P<0.001\)), and decreases in triglyceride and body weight (\(P<0.05\)) as compared with Ctrl (Table 1). No significant differences were observed between NxPD and NxPM. Total cholesterol was not statistically different across the three groups. No rat died during the study.

Peritoneal membrane function
D/P-Cre increased significantly in NxPD (1.04±0.02 vs. 0.43±0.03 in Ctrl; \(P<0.001\)) (Figure 4). This increase was significantly suppressed in NxPM (0.78±0.03 vs. 1.04±0.02 in NxPD; \(P<0.001\)). D/P-UN increased significantly in NxPD (1.07±0.03 vs. 0.66±0.03 in Ctrl; \(P<0.001\)) (Figure 4). This increase was significantly suppressed in NxPM (0.88±0.03 vs. 1.07±0.03 in NxPD; \(P<0.001\)).

Peritoneal membrane morphology
Uremia and PD induced submesothelial fibrosis (Figure 5A). The SML thickness increased approximately 4.4-fold in NxPD (\(P<0.001\) vs. Ctrl) (Figure 5B). Oral PM had no significant effects on SML thickening (Figures 5B).

The blood and lymphatic vessel densities in the mesenterium increased in NxPD (Figure 6). The blood vessel density increased 4.6-fold (\(P<0.001\)) (Figure 6B) and the lymphatic vessel density 10-fold (\(P<0.005\)) (Figure 6C). Oral PM significantly reduced the increases in the blood and lymphatic vessel densities (\(P<0.001\) and \(P=0.004\), respectively, vs. NxPD).
The blood vessel density in the SML of the parietal peritoneum significantly increased in NxPD ($P<0.001$ vs. Ctrl) (Figures 7A and B). Oral PM had no significant effects on this increase (Figures 7A and B).

Immunohistochemistry on the parietal peritoneum revealed about a 12-fold increase in the VEGF-positive cell density in SML in ($P<0.001$ vs. Ctrl) (Figures 8A and B). Oral PM suppressed the increase in the VEGF-positive cell density in SML; however, the difference did not reach statistical significance ($P=0.076$ vs. NxPD).

**Correlations between peritoneal membrane function and morphological parameters**

All groups were analyzed for correlations between D/P-Cre and morphological parameters (Table 2). D/P-Cre had significant positive correlations with the VEGF-positive cell density in SML, and the lymphatic vessel density and the blood vessel density in the mesenterium. Neither the blood vessel density in SML nor SML thickness had significant correlations with D/P-Cre.
DISCUSSION

We demonstrated that PM given orally to uremic rats on PD crossed the peritoneum with such efficiency that the PM concentration in the dialysate effluent was comparable to that in the circulation within one hour of dwell time. The activity of the intraperitoneal PM was quenched, as we intended, by the MG loading to the dialysate, as evident in the lack of effects on the pentosidine accumulation in the mesenterium or dialysate effluent. Oral PM suppressed pentosidine accumulation in the circulation and the increases in mesenteric vessel densities and small solute transport, but had no effects on serum creatinine or urea levels. Thus, reducing ROCs in the circulation alone suppressed morphological and functional deterioration of the peritoneum in uremic rats on PD, although the effects were insufficient to preserve adequate PD efficacy.

In order to reveal the effects of PM in the circulation, we had to have measures to quench PM in the peritoneal cavity because it was unknown if PM could cross the peritoneum. We loaded PD solution with 10 mmol/L of MG, a major glucose-derived RCO present in conventional heat-sterilized glucose-based dialysates (13, 26). This concentration is approximately 10,000 times its concentration in conventional dialysates (13, 25). Studies have shown that dialysate loading with up to 20 mmol/L of MG is required to induce full-scale morphological changes in the mesenterium by 21 days of daily dialysis in rats (25-27). Thus designed our experimental settings induced typical PD-associated morphological alterations of the peritoneum, and oral PM had no effects on pentosidine accumulation in the mesenterium or dialysate effluent despite the fact that PM crossed the peritoneum and amounted to some 20 μg/mL in the dialysate effluent. The MG loading caused as much peritoneal injury as expected and quenched PM transferred in the peritoneal cavity as effectively as intended.

The beneficial effects of oral PM on peritoneal morphology largely agree with those of intraperitoneal PM (24). Either intraperitoneal or oral administration of PM suppressed mesenteric neovascularization and the increase in small solute transport, resulting in significant correlations between D/P-Cre and the mesenteric vessel density with no effects on SML thickening (24). PD-associated functional deterioration is attributed to morphological changes of the peritoneal membrane, including increased vascular density and surface area and SML thickening (3-8). In humans, functional membrane deterioration correlates with increased vascular density and area,
“effective peritoneal surface area”, more strongly than with SML thickening (5, 7, 8). In uremic rats on PD, genetically-induced inhibition of angiogenesis suppressed mesenteric neovascularization and preserved the membrane function without reducing SML thickening, whereas genetic inhibition of transforming growth factor-β suppressed SML thickening with no effects on neovascularization or the membrane dysfunction (29). Similar results were obtained with VEGF neutralizing antibody in hyperglycemia-associated peritoneal injury and dysfunction in rats (30). Our present findings provide a further support to the notion that the increased vascular surface area, but not SML thickening, is the major morphological culprit of PD-associated peritoneal dysfunction.

Oral PM differs from intraperitoneal PM in the impact on the expression of VEGF, a growth factor implicated in PD-associated neovascularization of the peritoneal membrane (18, 31). Intraperitoneal PM significantly suppressed VEGF mRNA overexpression (24). Oral PM reduced the increase in the VEGF-positive cell density, but not to statistical significance ($P=0.076$). On the other hand, all-group analysis found a significant correlation between D/P-Cre and the VEGF-positive cell density as well as the mesenteric vessel density. Thus, VEGF is associated with mesenteric neovascularization even at the level of the individual variations in VEGF expression and the peritoneal morphology and function with or without experimental manipulations. Together with the fact that oral PM had no effects on the increases in creatinine and urea levels, our findings indicate two possibilities. First, circulating RCOs contributed little to peritoneal injury as compared to the considerably high carbonyl stress we applied to quench PM in the peritoneal cavity. Second, PM was either cleared from the circulation or consumed so soon that PM quenched only a small fraction of circulating RCOs. Further investigation is warranted with an unmodified dialysate and various dosages and timing of administration to identify the full potential of oral PM administration.

Because the diffusion of circulating RCOs into the peritoneal cavity was demonstrated, we have extended the carbonyl stress hypothesis and coined the word, “peritoneal carbonyl stress”, to describe carbonyl stress originating from both uremic circulation and dialysates (9, 11). This hypothetical mechanism opens three therapeutic approaches to protect the peritoneal membrane against the consequences of long-term PD. One is to inactivate glucose-derived RCOs in the dialysate by adding RCO
scavengers to the dialysate, or using glucose-free fluids or the multi-compartment bag system. Another is to scavenge RCOs in the circulation by oral administration of RCO scavengers. The third and most clinically practical approach is oral administration of RCO scavengers that can cross the peritoneum with high efficiency. PM is an RCO scavenger with its therapeutic values proven in a rat model of PD-associated peritoneal deterioration (24). We now demonstrated that orally given PM could cross the peritoneum with high efficiency. Application of 5 μg/mL of PM via the dialysate brought about significant protection (24). Our present study administered 50 mg of PM orally and achieved some 20 μg/mL in both the circulation and dialysate effluent within one hour of dwell time, which would have been sufficiently protective if unmodified dialysates were used. PM is a strong candidate for an oral medication against peritoneal carbonyl stress.

It should be noted that aminoguanidine, a scavenger of glucose degradation products, has been tested via dialysate in intact rats on PD and found to reduce peritoneal AGE accumulation and neovascularization (31). Unlike PM, however, aminoguanidine traps pyridoxal and carries safety concerns (31). In contrast, PM has advanced to phase 2 clinical trials in humans with type 2 diabetic nephropathy and has been found beneficial and safe (32). Furthermore, PM has been shown to inhibit advanced lipoxidation reactions (33). Lipid-derived RCOs diffuse into the peritoneal cavity, form advanced lipoxidation end products and contribute to peritoneal injury (11). Oral PM is likely to reduce lipid-derived RCOs in the circulation, which would be of additional therapeutic value.

Circulating RCOs diffuse into the peritoneal cavity due to osmotic gradient. This influx occurs in uremic subjects and continues until osmotic gradient dissipates. In fact, uremia per se can induce functional and morphological alterations of the peritoneal membrane in chronic renal failure patients (4) and rats (6). In contrast, glucose-derived RCOs in the dialysate inflict peritoneal injury primarily for the duration of dwell time and they appear to decay during dwell time (34). In addition, PD solutions containing less glucose degradation products and RCOs than conventional solutions have been developed (35-38). Combined use of these PD solutions and oral PM would reduce the risk of peritoneal injury and dysfunction in PD patients.
CONCLUSION

We show that orally given PM can reach the circulation and peritoneal cavity in uremic rats on PD, reduce carbonyl stress in the circulation and decelerate progressive morphological and functional deterioration of the peritoneum. Although these effects seen in the present settings were insufficient to maintain appropriate PD efficiency, our findings highlight the potential of PM to be an oral medication that can reduce carbonyl stress in the peritoneal cavity as well as in the circulation and protect against peritoneal injury and dysfunction in long-term PD.

Acknowledgments:
This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from the Japanese Association of Dialysis Physicians. Peritoneal dialysate was provided by Baxter Ltd. (Tokyo, Japan). We thank the Tokai University Education and Research Support Center, Ms. Yukiko Seki and Mr. Kentaro Toriumi for their excellent technical support, and Dr. Toshio Homma for editing the manuscript.

Conflict of interest:
T.K. has received honoraria from Kyowa Hakko Kirin, Chugai Pharmaceutical and Bayer Japan. H.K. has received honoraria from Kyowa Hakko Kirin and Chugai Pharmaceutical. M.F. has acted as a consultant for Kyowa Hakko Kirin, Bayer Japan and Novartis, and has received honoraria from Kyowa Hakko Kirin, Chugai Pharmaceutical, Bayer Japan, Novartis, Genzyme and Abbot Japan and grants/research support from Kyowa Hakko Kirin, Chugai Pharmaceutical and Bayer Japan. The other authors declare no conflict of interests.
REFERENCES
21. Booth AA, Khalifah RG, Todd P, Hudson BG. In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs): Novel inhibition of


2783-2792.


35. Schalkwijk CG, ter Wee PM, Teerlink T. Reduced 1,2-dicarbonyl compounds in bicarbonate/lactate-buffered peritoneal dialysis (PD) fluids and PD fluids based on glucose polymers or amino acids. Perit Dial Int 2000; 20: 796-798.


Table 1 Blood biochemical data and body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma creatinine (mg/dL)</th>
<th>Plasma urea (mg/dL)</th>
<th>Total cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>0.25±0.02</td>
<td>19.57±1.13</td>
<td>68.86±6.44</td>
<td>204.57±22.50</td>
<td>500.7±36.5</td>
</tr>
<tr>
<td>NxPD</td>
<td>0.76±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.50±13.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87.87±8.66</td>
<td>116.50±44.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>403.1±43.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NxPM</td>
<td>0.73±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.14±20.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.00±25.50</td>
<td>79.71±22.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>454.2±16.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Conversion factors are; creatinine in mg/dL to μmol/L, ×88.4; urea in mg/dL to mmol/L, ×0.357; total cholesterol in mg/dL to mmol/L, ×0.0259; triglycerides in mg/dL to mmol/L, ×0.01129.

Abbreviations: Ctrl, sham-operated rats; NxPD, subtotally nephrectomized rats with peritoneal dialysis; NxPM, subtotally nephrectomized rats with peritoneal dialysis and oral PM administration.

<sup>a</sup> P<0.001 vs. Ctrl; <sup>b</sup> P<0.05 vs. Ctrl.
Table 2 Correlations of dialysate-to-plasma ratio of creatinine with morphological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-positive cell density in the SML</td>
<td>0.72</td>
<td>0.014</td>
</tr>
<tr>
<td>Lymphatic vessel density in the mesenterium</td>
<td>0.71</td>
<td>0.019</td>
</tr>
<tr>
<td>Blood vessel density in the mesenterium</td>
<td>0.67</td>
<td>0.035</td>
</tr>
<tr>
<td>Blood vessel density in the SML</td>
<td>0.65</td>
<td>0.053</td>
</tr>
<tr>
<td>SML thickness</td>
<td>0.55</td>
<td>0.173</td>
</tr>
</tbody>
</table>

Correlations of dialysate-to-plasma ratio for creatinine with each one of the indicated parameters were analyzed by Pearson’s correlation test using the data from all three groups.

Abbreviations: SML, submesothelial matrix layer; VEGF, vascular endothelial growth factor.
Figure legends

Figure 1 Experimental protocol
Seven-week-old rats were subjected to sham-operation alone (Ctrl), subtotal nephrectomy (5/6 Nx) with 3 weeks of peritoneal dialysis (PD) using a 3.86% glucose-based fluid supplemented with 10 mmol/L methylglyoxal (MG) (NxPD) or subtotal nephrectomy with PD and daily gavage of 50 mg pyridoxamine (PM) (NxPM). At the end of experiment, peritoneal equilibration test was performed, and blood, dialysis effluent and tissues were sampled.

Figure 2 Pyridoxamine concentrations in the circulation and dialysate effluent
At the end of the experiment, sham-operated rats without peritoneal dialysis (Ctrl) and subtotally nephrectomized rats on peritoneal dialysis received gavage of vehicle (NxPD) or 50 mg pyridoxamine (NxPM) and, one hour later, dialysate (30 mL) was infused. After one hour of dwelling, dialysate effluent and blood were collected, and pyridoxamine concentration was determined as described in Methods. * P<0.001 vs. other groups.

Figure 3 Pentosidine content
Pentosidine content was determined at the end of the experiment in (A) mesenterium, (B) dialysate effluent and (C) blood as described in Methods in sham-operated rats without peritoneal dialysis (Ctrl), subtotally nephrectomized rats with peritoneal dialysis (NxPD), and subtotally nephrectomized rats with peritoneal dialysis and oral pyridoxamine (NxPM).

Figure 4 Dialysate-to-plasma ratios for creatinine and urea
At the end of the experiment, peritoneal equilibration test (60 minutes) was performed on the day of sacrifice in sham-operated rats without peritoneal dialysis (Ctrl), subtotally nephrectomized rats with peritoneal dialysis (NxPD), and subtotally nephrectomized rats with peritoneal dialysis and oral pyridoxamine (NxPM) as described in Methods. Dialysate-to-plasma ratios for creatinine (D/P-Cre) and urea (D/P-UN) are shown.
**Figure 5** Peritoneal membrane morphology
A, HE-stained sections of the parietal peritoneum of sham-operated rats without peritoneal dialysis (Ctrl), subtotally nephrectomized rats with peritoneal dialysis (NxPD), and subtotally nephrectomized rats with peritoneal dialysis and oral pyridoxamine (NxPM). B, The thickness of the submesothelial matrix layer (μm). [The bars in the photos indicate 100 μm. HE staining, thickness 5 μm, magnification ×100]

**Figure 6** Blood and lymphatic vessels in the mesenterium
A, Immunostaining for blood vessels (red arrows) and lymphatic vessels (blue arrows) in the mesenterium of sham-operated rats without peritoneal dialysis (Ctrl), subtotally nephrectomized rats with peritoneal dialysis (NxPD), and subtotally nephrectomized rats with peritoneal dialysis and oral pyridoxamine (NxPM). B, Blood vessel density of the mesenterium. C, Lymphatic vessel density of the mesenterium. [The bars in the photos indicate 100 μm. HE staining, thickness 5 μm, magnification ×200]

**Figure 7** Blood vessels in the submesothelial matrix layer
A, Immunostaining for blood vessels (arrows) in the submesothelial matrix layer of the parietal peritoneum of sham-operated rats without peritoneal dialysis (Ctrl), subtotally nephrectomized rats with peritoneal dialysis (NxPD), and subtotally nephrectomized rats with peritoneal dialysis and oral pyridoxamine (NxPM). B, Blood vessel density of submesothelial matrix layer. [The bars in the photos indicate 100 μm. HE staining, thickness 5 μm, magnification ×100]

**Figure 8** Vascular endothelial growth factor expression in the mesenterium
A, Immunostaining for vascular endothelial growth factor (VEGF)-positive cells (arrows) in the submesothelial matrix layer of the parietal peritoneum of sham-operated rats without peritoneal dialysis (Ctrl), subtotally nephrectomized rats with peritoneal dialysis (NxPD), and subtotally nephrectomized rats with peritoneal dialysis and oral pyridoxamine (NxPM). B, VEGF-positive cell density of the submesothelial matrix layer. [The bars in the photos indicate 100 μm. HE staining, thickness 5 μm, magnification ×200]
<table>
<thead>
<tr>
<th>Weeks</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham operation</td>
<td>Standard food</td>
<td>Sham operation</td>
<td>No PD</td>
<td>Peritoneal equilibration test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NxPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/6 Nx</td>
<td>3.86% glucose dialysate + 10 mmol/L MG</td>
<td>3.86% glucose dialysate + 10 mmol/L MG</td>
<td>PM once daily</td>
<td>Blood and tissue sampling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NxPM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1
Figure 2

Pyridoxamine (ng/mL)

Ctrl  NxPD  NxPM

Blood  Dialysate effluent

*  *
Figure 3

A. Mesenterium pentosidine (pmol/mg)

- Ctrl
- NxPD
- NxPM

B. Dialysate effluent pentosidine (pmol/mg)

- Ctrl
- NxPD
- NxPM

C. Plasma pentosidine (pmol/mg)

- Ctrl
- NxPD
- NxPM

P<0.001
P<0.001
NS

P=0.018
P=0.041
NS

P=0.039
P=0.018
Figure 4

D/P-Cre

P<0.001  P<0.001  P<0.001

Ctrl  NxPD  NxPM

D/P-UN

P<0.001  P<0.001

Ctrl  NxPD  NxPM
Figure 5

A

Ctrl

NxPD

NxPM

B

Thickness (μm)

P<0.001

P<0.001

NS

Ctrl

NxPD

NxPM
Figure 6

A  Ctrl  NxPM  NxPD

B

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>NxPD</th>
<th>NxPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>density (per mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>=0.006</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>NxPD</th>
<th>NxPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph vessel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>density (per mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>=0.004</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7

A

Ctrl

NxPD

NxPM

B

Blood vessel density (per mm²)

P<0.001

P<0.001

NS

Ctrl

NxPD

NxPM
Figure 8

A

Ctrl

NxPD

NxPM

B

VEGF-positive cells (per mm²)

P<0.001

P<0.001

P=0.076

Ctrl

NxPD

NxPM