**Evaluation of Renal Microcirculation by Contrast-Enhanced Ultrasound With Sonazoid™ as a Contrast Agent**

**Comparison Between Normal Subjects and Patients With Chronic Kidney Disease**

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**Summary**

Chronic kidney disease (CKD) is a major and serious risk factor for cardiovascular disease (CVD). Continuous hypoxia due to hypoperfusion in peritubular capillaries is one of the factors aggravating CKD, but evaluation of perfusion in this region is difficult using clinically available imaging methods. Since the second-generation ultrasound contrast agent Sonazoid™ has a stable shell, it enables visualization of the renal vasculature for a long period of time. We therefore evaluated changes in contrast-enhanced ultrasound (CEUS) imaging with Sonazoid™ in CKD patients.

Sonazoid™ was used in 85 CKD patients and 5 control subjects, and images were recorded for 10 minutes. Time-intensity curves were generated from the images of 62 time points in both cortex and medulla. In control samples, contrast enhancement spread from the hilar portion to the periphery along the direction of arterial flow, and renal cortex and medulla were then enhanced in sequence. Enhancement was maximal soon after, then gradually decreased, but was still visible at 600 seconds. In CKD patients, renal contrast enhancement was attenuated in both cortex and medulla. On time-intensity curves, the attenuation of enhancement was composed of delayed rising, reduction of peak, and acceleration of decay in both cortex and medulla with progression of renal dysfunction. No side effects of the contrast agent were observed in any subjects.

The attenuation of renal contrast enhancement observed in CKD patients appears to reflect disturbance of perfusion in peritubular capillaries. CEUS with Sonazoid™ is a useful and safe means of visualizing the renal microvasculature. (Int Heart J 2010; 51: 176-182)

**Key words:** Chronic kidney disease (CKD), Cardiovascular disease (CVD), Cardio-renal correlation, Renal microcirculation, Contrast-enhanced ultrasound (CEUS), Second-generation contrast agent, Visualization of renal vasculature, Renal cortex, Renal medulla, Time-intensity curve

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Chronic kidney disease (CKD) is a major and serious risk factor for cardiovascular disease (CVD). In fact, mortality and morbidity from CVD have been shown to be high in CKD even from its early stage. Therefore, it is important to find CKD in its early stage and to properly manage it in order to prevent progression to end-stage kidney disease or the development of CVD.

The cardio-renal correlation depends on the risk factors of CKD and CVD which are closely related to each other, causing organ failure through vascular endothelial damage and arteriosclerosis. In CKD, oxidative stress is caused by many factors, for example, urine toxins, the renin-angiotensin-aldosterone system, and chronic hypoxia. Continuous hypoxia in the renal tubulointerstitium is known to be one of the major risk factors accelerating the progression of renal dysfunction and thus leads to development of CVD. Hypoxia in the kidney is induced by the loss of peritubular capillaries in the late stage of CKD. Thus, in this study, we aimed to evaluate the dynamics of the renal microcirculation using contrast-enhanced ultrasonography and to determine how it varied depending on the CKD stage.

Evaluation of renal circulation is thus important in the management of CKD and CVD. However, evaluation of blood flow in this region is difficult using clinically available imaging studies. Sonazoid™ (Daiichi-Sankyo, Tokyo) is used as a second-generation ultrasound contrast agent, and consists of an aqueous dispersion of lipid-stabilized perfluorobutane-filled gas micro-bubbles with a median volume diameter of approximately 3 μm. Since the micro-
bubbles of the second-generation echo enhancers are stabilized with a shell of a phospholipid monolayer, this agent has proven useful for characterization of the vasculature over prolonged periods of observation. Since perfluorobutane is excreted almost entirely by exhalation, Sonazoid™ can be used in patients with renal dysfunction without any harmful effects. However, there have been few reports on contrast-enhanced ultrasound (CEUS) imaging studies using second-generation echo enhancers in human kidney. To determine the usefulness of CEUS in the visualization of peritubular capillaries and changes in enhancement with progression of renal dysfunction, we measured renal perfusion by CEUS with administration of Sonazoid™ in normal subjects and patients with chronic kidney disease, and evaluated the correlations between glomerular filtration rate and enhancement in the renal cortex and medulla.

Methods

Subjects: Patients with chronic kidney disease (CKD) scheduled for routine renal ultrasound (US) study in our clinic between November 2007 and June 2008 were consecutively enrolled. The following exclusion criteria were used: egg allergy, severe heart or pulmonary disease, pregnancy, and distinct difference in sizes of the two kidneys (more than 12 mm). Five volunteers without kidney disease were also examined as control subjects. The present study was approved by the Institutional Committee on Human Research of St. Marianna University School of Medicine (No. 1303). Written informed consent was obtained from all patients and normal volunteers prior to enrollment.

US examination protocol: We performed US with an ultrasound scanner (Aplio TA700; Toshiba, Tokyo) using an electrical convex 3.5 MHz probe in conventional and harmonic modes. The CEUS examination was performed using contrast harmonic imaging at a CHI gain of 65-67 dB, mechanical index of 0.22-0.25, sensitivity time control placed at center, and focus placed at 4-6 cm. The frame rate was 15 frames per second.

All subjects were scanned with a lateral or translumbar subcostal approach to the kidney in the supine position without any special preparation. While visualizing the long-axis view of the kidney, 0.0075 mL/kg of Sonazoid™ was administered as a bolus intravenous injection from the cannulated medial cubital vein. The observed kidney was selected as the easier of the two kidneys to visualize. Enhanced imaging was recorded for 10 minutes after the injection of Sonazoid™. All imaging before and during enhancement was recorded continuously on the hard disk of the US scanner for subsequent analysis.

Analysis of contrast medium dynamics: Images at 62 time points - every second for the first 45 seconds, every 15 seconds for the next 75 seconds, every 30 seconds for the next 180 seconds, and then every 60 seconds till 600 seconds - were selected and examined using MITANI WinROOF™ image analysis software (Mitani Corporation, Tokyo). Three 20 × 20 pixel regions-of-interest (ROIs) each in cortex and medulla, and time-intensity curves were generated.

Table 1. Points of Analysis and Characterization of Time-Intensity Curves for Cortex and Medulla

- **Time points**
  - Injection point: time at which Sonazoid™ was injected
  - Baseline point: time just before the start of enhancement
  - Start point: time at which intensity in the ROI had increased more than 1 dB in grayscale above the baseline point
  - Peak point: time at which maximum intensity was reached
  - Half maximal point: time at which intensity was decreased by half compared with that at peak

- **Periods of time**
  - S-P period: the interval from the start point to the peak point
  - P-H period: the interval from the peak point to the half-maximal point

- **Slopes**
  - S-P slope: the gradient of intensity from the start point to the peak point; rate of increase in enhancement
  - P-H slope: the gradient of intensity from the peak point to the half-maximal point; rate of decrease in enhancement

Figure 1. Enhancement image from a normal control. Echogenicities after a bolus injection of Sonazoid™ were measured serially in two or three 20 × 20 pixels of regions of interest (ROIs) each in cortex and medulla, and time-intensity curves were generated.

Figure 2. Schematic illustration of the cortical time-intensity curve and analysis scheme. The medullary time-intensity curve was analyzed in the same fashion as the cortical curve.
ured serially in each ROI and time-intensity curves were generated. Intensity was a measure of the echogenicity defined as the mean of the gray-scale values within the ROI. Time-intensity curves were examined for the parameters indicated in Table I and Figure 2.

Statistical analysis: All values are the mean ± SD. Statistical examination of variables was performed by one-way analysis of variance, and correlations were calculated using least-squares fit linear regression analysis with Jump software™ (SAS Institute Inc, Cary, NC, USA). Values of \( P < 0.05 \) were considered significant.

RESULTS

Patient profiles: CEUS was performed in 85 chronic kidney disease patients (60 males and 25 females) ranging in age from 21 to 85 years (mean, 56.7 ± 17.1). Their serum creatinine was 2.92 ± 2.78 mg/dL (range, 0.60 - 13.66), urinary protein excretion 2.30 ± 3.08 g/g \( \cdot \) Cr (range, 0 - 12.7), and renal long axis 104.7 ± 12.0 mm (range, 72.7 - 134.6). The left kidney was used in 81 subjects, while the right kidney was used in 4 subjects.

Among the 85 patients, 80 patients were diagnosed based on histology or clinical findings, and include 29 cases of diabetic nephropathy, 29 of chronic glomerulonephritis, 7 of chronic tubular-interstitial nephritis, 7 of nephrotic syndrome, 6 of benign nephrosclerosis, and one each of light-chain deposition disease and medullary nephropathies. Five cases were of unknown etiology.

Patients were classified into 4 groups by CKD stage according to the creatinine-based estimate of glomerular filtration rate (eGFR) as follows: \(^{15}\) 17 patients in CKD stage 1 + 2 (eGFR greater than 60 mL/min), 28 patients in CKD stage 3 (30 - 59 mL/min), 14 patients in CKD stage 4 (15 - 29 mL/min), and 26 patients in CKD stage 5 (less than 15 mL/min). Table II shows the basic parameters in the control subjects and the CKD patients.

No side effects of the contrast agent were observed in any subject, and no aggravation of urinary or hematological findings were detected during the 3-month period after the study.

Enhanced imaging of the kidney in control subjects: Shortly after the injection of contrast agent, enhancement in control subjects began from the hilar portion of the kidney, and then spread peripherally along the interlobar arteries, arcuate arteries, and interlobular arteries in sequence (Figure 3). After that, the renal cortex was enhanced from the side of the renal capsule to the renal pyramid. Enhancement of the renal cortex was maximal 15 to 20 seconds after the injection of contrast enhancer (Figure 4). A few seconds after the start of cortical enhancement, the renal medulla was also enhanced from the cortical side to the inner medulla. Enhancement of the renal medulla was maximal 10 to 20 seconds after maximal enhancement of the cortex. Contrast enhancement then decreased gradually, and disappeared in 10 to 12 minutes in both the cortex and medulla (Figure 5).

### Table II. Basic Parameters in the Control Subjects and CKD Patients

<table>
<thead>
<tr>
<th>CKD</th>
<th>Control subjects (( n = 5 ))</th>
<th>Stage 1+2 (( n = 17 ))</th>
<th>Stage 3 (( n = 28 ))</th>
<th>Stage 4 (( n = 14 ))</th>
<th>Stage 5 (( n = 26 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal size (mm)</td>
<td>111.6 ± 7.93</td>
<td>109.3 ± 8.61</td>
<td>107.5 ± 11.15</td>
<td>104.7 ± 13.31</td>
<td>97.4 ± 11.94</td>
</tr>
<tr>
<td>ILA PSV (cm/s)</td>
<td>32.1 ± 5.04</td>
<td>25.4 ± 6.15</td>
<td>21.7 ± 6.63</td>
<td>29.5 ± 14.48</td>
<td>21.0 ± 9.58</td>
</tr>
<tr>
<td>ILA EDV (cm/s)</td>
<td>12.6 ± 2.94</td>
<td>9.5 ± 2.79</td>
<td>7.6 ± 3.7</td>
<td>6.1 ± 4.51</td>
<td>4.1 ± 1.58</td>
</tr>
<tr>
<td>ILA RI</td>
<td>0.61 ± 0.07</td>
<td>0.63 ± 0.05</td>
<td>0.65 ± 0.08</td>
<td>0.79 ± 0.07</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>UP (g/gCr)</td>
<td>0.61 ± 0.07</td>
<td>0.63 ± 0.05</td>
<td>0.65 ± 0.08</td>
<td>0.79 ± 0.07</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>13.2 ± 1.30</td>
<td>13.0 ± 2.28</td>
<td>9.9 ± 1.42</td>
<td>8.6 ± 1.38</td>
<td>3.2 ± 2.81</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126 ± 20.5</td>
<td>129 ± 18.3</td>
<td>143 ± 17.0</td>
<td>144 ± 18.9</td>
<td>132 ± 12.9</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81 ± 11.9</td>
<td>78 ± 13.9</td>
<td>75 ± 14.6</td>
<td>76 ± 27.9</td>
<td>70 ± 20.5</td>
</tr>
<tr>
<td>Past disease: DM (%)</td>
<td>0</td>
<td>12</td>
<td>11</td>
<td>64</td>
<td>58</td>
</tr>
</tbody>
</table>

Mean ± SD. ILA indicates interlobar arteries; PSV, peak systolic velocity; EDV, end-diastolic velocity; RI, resistive index (RI = PSV - EDV/PSV); UP, urinary protein; SBP, systolic blood pressure; DBP, diastolic blood pressure; and DM, diabetes mellitus.

![Figure 3. Representative enhancement images with early-phase real-time pulse inversion after a bolus injection of Sonazoid™ in control subjects. (a) 8 seconds after injection; baseline point, (b) 8.5 seconds; start point of renal enhancement; contrast agent influx into segmental arteries, (c) 9.0 seconds; influx into interlobar arteries, (d) 9.2 seconds; influx into arcuate arteries, (e) 9.8 seconds & (f) 10.0 seconds; influx into interlobular arteries toward the subcapsular renal cortex, (g) 10.2 seconds; start point of cortical enhancement, (h) 10.4 seconds · (i) 10.5 seconds · (j) 10.6 seconds; spreading of contrast agent serially throughout cortex, (k) 11.2 seconds; start point of medullary enhancement, (l) 13 seconds; maximal intensity of cortical enhancement.](image)
**Figure 4.** Representative serial contrast enhancement images in early cortical phase in control and CKD stage 5 subjects. In CKD stage 5, the start point and augmentation of cortical enhancement were delayed compared to normal control. Visualization of interlobular arteries decreased with poorly-defined margins in CKD stage 5. Efflux of Sonoazoid\textsuperscript{TM} from cortex and medulla was accelerated in CKD stage 5.

**Figure 5.** Representative serial contrast enhancement images throughout the period of observation in control and CKD stage 5 subjects. In CKD stage 5, the start point of medullary enhancement was delayed and maximal intensities of enhancement in cortex and medulla were both reduced compared to normal control. Efflux of Sonoazoid\textsuperscript{TM} from cortex and medulla was accelerated in CKD stage 5.

**Enhanced imaging of the kidney in advanced CKD:** Serial images of a representative case of CKD stage 5 disease are shown in Figures 4 and 5. In advanced CKD, the interlobar and interlobular arteries were visualized for a longer period of time, and visualization of the interlobular arteries was poorer. The echogenicity of enhancement in both cortex and medulla was less and the rate of decrease in echogenicity was more rapid than in controls, and enhancement disappeared in 3 to 5 minutes (Figure 5).

**Analysis of time-intensity curves in cortex and medulla:** On analysis of the time-intensity curve for the controls, renal cortex exhibited peak echogenicity in about 10 seconds after the start of cortex enhancement, as determined by an increase in echogenicity of more than one dB grayscale compared with baseline. After the peak, intensity decreased once, and then a small second peak was observed in all control subjects (Figure 6). There was only one peak in the medulla, which was later and smaller than the first peak in cortex. The rate of attenuation of intensity was almost the same in cortex and medulla. Intensity decreased with progression of CKD stage, as shown by the time-intensity curves for each group (Figure 7). A decrease in magnitude of the initial rise, delayed interval to the peak point, decrease in peak intensity, and early decrement in both cortex and medulla were observed with progression of CKD stage. To examine the initial rise in intensity, we compared the rising slope from the start point to the peak point (S-P slope) in both cortex and medulla (Figure 2). In the cortex, the S-P...
slope was significantly decreased in stage 5 compared with the control group and stages 1 + 2 (Figure 8a). In contrast, in the medulla, a slight decrease in the S-P slope with the progression of the CKD stages was not seen (Figure 8b).

There was also a slight decrease in peak intensity with progression of CKD stage. Peak intensity was significantly less in stage 5 than in stages 1 + 2 in both cortex and medulla (Figure 9).

Next, the decrease following peak echogenicity was examined by measurement of decrease in slope from the peak point to the half-maximal point (P-H slope) in both cortex and medulla. There was a slight increase in P-H slope with progression of CKD stage, and P-H slope was significantly higher in stage 5 than in the control, stage 1 + 2, and stage 3 groups in both cortex and medulla (Figure 10).

Correlations between echogenicity parameters and eGFR: All three parameters of the time-intensity curve, S-P slope, peak intensity, and P-H slope, exhibited significant correlation with eGFR in both cortex and medulla (Table III). Among them, P-H slope in the medulla exhibited the strongest correlation with eGFR.

**Discussion**

In this study we demonstrated that CEUS imaging with Sonazoid® enabled direct visualization of flow in the renal vasculature, including peritubular capillaries. We also demonstrated that contrast enhancement in both cortex and medulla decreased with progression of CKD stage. To the best of our knowledge, this is the first report of renal CEUS study with Sonazoid® in human CKD.

Almost all previous studies of renal vasculature with
CEUS used first-generation contrast agents, which elicit signals by the collapse of micro-bubbles, and the observation period was too short for clear examination of the renal microvasculature. In contrast, the second-generation enhancer Sonazoid™ has a stable phospholipid shell in each micro-bubble, and since the enhancement signals obtained with it are elicited by resonance rather than bursting of micro-bubbles, stable long-term contrast enhancement can be obtained. Although Sonazoid™ is known to be captured by the reticulo-endothelial system, after intravenous injection almost all of it remains entirely within the intravascular space without being trapped in normal small vessels. Furthermore, Sonazoid™ is rheologically similar to red blood cells. These characteristics of Sonazoid™ in renal CEUS imaging enable prolonged visualization of blood flow in the renal arterioles and capillaries, as demonstrated in this study.

In normal subjects, contrast enhancement spread from the hilar portion to the periphery. After that, the renal cortex and, a few seconds later, renal medulla were enhanced. This pattern of enhancement indicated that after injection, Sonazoid™ reached the glomeruli through normal anatomical vessels, and was then distributed in the cortex and medulla through the peritubular capillaries. Analysis of echogenicity in ROIs in the cortex indicated that there was a second small peak following the first peak in the control subjects. The interval between these peaks was about 20 seconds, a period coinciding with the time required for systemic circulation.

The present study demonstrated that in CKD patients renal contrast enhancement was attenuated with deterioration of renal function. Analysis of captured ultrasound images showed that this attenuation consisted of a delay in the rise in echogenicity, reduction of peak intensity, and acceleration of decay of enhancement in both cortex and medulla. We analyzed these factors using the S-P slope, peak intensity, and P-H slope, respectively, and found that the S-P slope and peak intensity were significantly decreased and the P-H slope significantly increased with progression of CKD compared with early-stage CKD. In addition, these values exhibited significant correlation with eGFR in both cortex and medulla. Hosotani, et al. found that the intensity of cortical contrast enhancement gradually decreased with a decline in renal function, with use of the first-generation contrast agent Levovist. They also found that this attenuation exhibited a significant positive correlation with renal plasma flow. Although their period of observation of cortical enhancement was only 7 seconds, the delay in rising demonstrated by a decrease in S-P slope and reduction of peak echogenicity with progression of CKD stage observed in the early phase of our present study with 600-second observation are consistent with their findings. It seems likely that the increase in renal resistance due to the decrease in number of glomeruli and peritubular capillaries as well as narrowing of arterioles contributes to the delay in rising and reduction of peak intensity with progression of CKD stage we observed. The attenuation of visualization of interlobular arteries and sluggish efflux of enhancement from interlobar arteries with progression of renal dysfunction are also consistent with this conclusion. Fisher, et al. examined the usefulness of CEUS with another second-generation ultrasound contrast medium, SonoVue®, in kidney transplant recipients, and found a delayed and small increase in enhancement in cortex in patients with acute tubular necrosis and vascular rejection.

The blood flow in CKD stage 4 seems high compared to that in CKD in other stages (Figures 8 and 9). However, the number of patients in CKD stage 4 was rather small (n = 14), the variations in the S-P slope in this stage were rather large, and there was no significant difference in the S-P slopes between CKD stage 4 and CKD in other stages. Therefore, we assumed that the high value in the S-P slope in CKD stage 4 had no clinical significance.

Delay in rising and reduction of peak in cortex thus appear to reflect reduction of flow in peritubular capillaries not only in chronic but also acute renal injury. Our study also demonstrated that decay from peak intensity accelerated with progression of CKD stage. The P-H intervals in cortex and medulla exhibited significant negative correlation with eGFR, which was especially strong in medulla. Since a large proportion of contrast agent remains entirely within the intravascular space, retention time appears to be proportional to total capillary volume. The decrease in number of nephrons and tubulo-interstitial injury in renal dysfunction are attributed to the reduction of peritubular capillary area, especially in those capillaries branching and connecting in parallel.

The acceleration of decay with worsening of eGFR observed in this study might thus indicate a decrease in the volume of postglomerular capillaries. We speculate that the anatomical vulnerability of the peritubular capillaries in the medulla to ischemia accounts for the strong correlation of P-H interval in medulla with eGFR.

However, it appears that the attenuation of contrast enhancement observed in CKD patients cannot be accounted for solely by a decrease in GFR. The S-P slope and peak intensity in cortex and medulla in CKD stage 4 were higher, though not to significant extents, than those in CKD stage 3. Therefore, to make renal CEUS with Sonazoid™ clinically useful, further investigation of the relationships between alteration of contrast enhancement and the degrees of tubulo-interstitial and arteriolar injury in renal biopsy specimens, degree of proteinuria, and markers of renal tubular injury will be needed. Sonazoid™ has been available for clinical use and no adverse events have thus far been reported. In the present study, no side effects were noted during the 3-month period following the study in any subjects. This study thus showed that Sonazoid™ can be safely used in CKD patients.

In conclusion, when using Sonazoid™ as a contrast agent, renal CEUS enabled visualization of flow in peritubular capillaries in cortex and medulla in patients with CKD. With progression of renal dysfunction, contrast enhancement of cortex and medulla decreased, and imaging analysis showed that this change was due to delay in rising, reduction of peak intensity, and acceleration of decay of enhancement. Renal CEUS with Sonazoid™ is useful and safe for visualization of the renal microvasculature in CKD patients. As CKD is a risk factor for CVD, further study is necessary to clarify the relationship between renal microcirculation and the systemic vascular damage in order to cope with CVD in the future.
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