Comparison of Histopathologic Analysis Following Renal Sympathetic Denervation Over Multiple Time Points

Kenichi Sakakura, MD; Stefan Tunev, DVM; Kazuyuki Yahagi, MD; Amanda J. O’Brien, DVM; Elena Ladich, MD; Frank D. Kolodgie, PhD; Robert J. Melder, ScD; Michael Joner, MD; Renu Virmani, MD

Background—The pathology of radiofrequency-derived sympathetic renal denervation has not been studied over time and may provide important understanding of the mechanisms resulting in sustained blood pressure reduction. The purpose of this study was to investigate chronological changes after radiofrequency-renal denervation in the swine model.

Methods and Results—A total of 49 renal arteries from 28 animals with 4 different time points (7, 30, 60, and 180 days) were examined. Semiquantitative histological assessment of arteries and associated tissue was performed to characterize the chronological progression of the radiofrequency lesions. Arterial medial circumferential injury (%) was greatest at 7 days (38±13%), followed by 30 days (31±6%) and 60 days (31±15%), and least at 180 days (21±12%) (P=0.046). Nerve injury score was significantly greater (P<0.001) at 7 days (3.9±0.4) compared with 30 days (2.5±0.5), 60 days (2.6±0.5), and 180 days (1.9±0.9). Tyrosine hydroxylase score, which assesses functional nerve damage, was significantly less after 7 (1±1) and 30 days (0.7±0.6) compared with 60 (2.7±0.6) and 180 days (2.7±0.6; P=0.01). Focal nerve regeneration at the sites of radiofrequency ablation was observed in 17% of renal arteries at 60 days and 71% of 180 days.

Conclusions—Nerve injury after radiofrequency ablation was greatest at 7 days, with maximum functional nerve damage sustained ≤30 days. Focal terminal nerve regeneration was observed only at the sites of ablation as early as 60 days and continued to 180 days. Renal artery and peri-arterial soft tissue injury is greatest in the subacute phase, and least in the chronic phase, suggesting gradual recovery of the renal arterial wall and surrounding tissue. (Circ Cardiovasc Interv. 2015;8:e001813. DOI: 10.1161/CIRCINTERVENTIONS.114.001813.)

Key Words: pathology ■ preclinical study ■ radiofrequency ■ renal artery ■ renal denervation

Hypertension is closely associated with cardiovascular morbidity and mortality,1 and the use of antihypertensive medications significantly effects the control of blood pressure. However, the prevalence of resistant hypertension, which is defined as failure to achieve blood pressure control with optimal antihypertensive drug usage of ≥3 medications, remains as high as 12% to 15%. 1–4 Radiofrequency renal sympathetic denervation was recently introduced as an innovative therapeutic option for the treatment of resistant hypertension and is associated with reduction in blood pressure in early nonrandomized clinical trials.5–7 Furthermore, long-term follow up of the Symplicity HTN-1 study demonstrated that blood pressure reduction was maintained ≤3 years in 93% of 88 patients who completed 3 year follow-up8 while the total number of patients who completed long-term follow-up remains small. However, there remains a clear gap between these clinical observations and our knowledge of the mechanisms involved in sustained blood pressure reduction as shown by the failure to meet the efficacy end point in the Symplicity HTN-3 study.9 Therefore, there is a clear need to refocus scientific efforts toward a better understanding of the treatment effects and to demonstrate its efficacy in both short- and long-term preclinical studies.

Preclinical animal studies allow us to evaluate treatment effects of this novel technology at the morphological and functional level. Although a small number of acute or chronic preclinical studies have been published on the effects of radiofrequency-based sympathetic denervation,10,11 a chronological systematic histopathologic investigation of treatment effects associated with radiofrequency ablation of peri-arterial renal sympathetic nerves is lacking. The main purpose of this study was to investigate the temporal sequence of vascular and peri-vascular changes after radiofrequency-based sympathetic denervation in a healthy swine model.

Methods

Renal Denervation System

The Symplicity renal denervation (RDN) system used in these studies consists of the Symplicity catheter and its generator as previously described.10 The Symplicity catheter is a single-use, 6F compatible...
WHAT IS KNOWN

• Radiofrequency renal sympathetic denervation is a promising new treatment option for patients experiencing resistant hypertension.
• Although a small number of acute or chronic preclinical studies have been published on the effects of radiofrequency-based sympathetic denervation, a chronological systematic histopathologic investigation of treatment effects associated with radiofrequency ablation of peri-arterial renal sympathetic nerves is lacking.

WHAT THE STUDY ADDS

• Nerve injury after radiofrequency renal denervation is greatest in the subacute phase (7 days) compared with the transitional phase (30 and 60 days) and chronic phase (180 days), with peak functional nerve damage seen at 30 days.
• Focal nerve regeneration was only observed at the sites of radiofrequency injury in the transitional phase (60 days) and was sustained to the chronic phase (180 days).
• Renal arterial injury is greatest in the subacute phase (7 days), and least in the chronic phase (180 days), suggesting complete healing of the arterial wall and soft tissue at this time point in the swine model.

Electrode-tipped Catheter specifically designed to deliver low-level radio frequency energy—through the vascular wall of the renal arteries. Typically 3 to 6 radiofrequency ablations (120 seconds) were performed within the main stem of each renal artery depending on the available treatable length as previously described. The first ablation was located 5 mm proximal to the bifurcation, and subsequent ablations were performed after repositioning 5 mm proximally and rotating it 90 degrees. The result was a spiral pattern of individual treatment sites spaced rotationally and longitudinally along the artery.

Animal Study

All aspects of the studies were approved by the Institutional Animal Care and Use Committees before study conduct (Lychron LLC [Mountain View, CA]; ISIS Services LLC [San Carlos, CA]).

To assess the effect of catheter-based radiofrequency energy delivery on the renal arteries, 3 to 6 ablations (average treatment per vessel: 4.1±0.8) were delivered to the renal arteries in 28 female or castrated male Yorkshire domestic swine. Table 1 lists the number of animals and treated arteries per time point. All except 180-days animals were treated bilaterally, while the 180-days animals were treated unilaterally. All animals received 81 mg of Aspirin single dose daily for ≥14 days after the procedure. Anticoagulation during the procedure was achieved with heparin to maintain a coagulation time ≥250 seconds. Each animal was positioned in dorsal recumbency in the operating room with a radio opaque ruler placed under the animal in the vicinity of the kidneys. A 6F or 7F introducer sheath was placed by percutaneous cannulation of the right femoral artery. Anatomic eligibility of the artery was determined by angiography before treatment.

To perform the RDN procedure, arteries were required to be ≥18 mm in length and 3.5 mm in diameter. Duplicated renal arteries or kidneys with large accessory renal arteries were excluded. The catheter was placed at the most distal treatment location, and the electrode tip was deflected toward the vessel wall. The catheter was rotated into position using fluoroscopic guidance and by monitoring impedance. Radiofrequency energy was applied for 120 seconds. The electrode tip was then straightened and the catheter positioned at the next treatment site 5 mm proximally, deflected toward the vessel wall and activated again.

Terminal angiography was performed at each termination time point, and the animals were heparinized and humanely euthanized. The renal arteries and attached kidneys were pressure perfusion fixed through the cannulated aorta after flushing with Lactated Ringers solution followed by perfusion fixation with 10% neutral buffered formalin. The tissue block, which included a portion of the kidneys, major vessels, ureters, renal lymph nodes, and any associated soft tissues, was dissected and immersed in 10% neutral buffered formalin. Also, the animals underwent a limited necropsy of the urinary system including renal vessels, kidneys, ureters, and adrenal glands, which did not show device-related pathology.

Histopathologic Processing

Renal arteries with surrounding tissue were cut from the ostium of renal artery to the renal hilum at 3 to 4 mm intervals with ≥13 segments examined from each artery (mean number of segments per artery: 8.4±1.8) and submitted in separate cassettes for dehydration and paraffin embedding. A total of 469 paraffin blocks from 28 pigs including eleven 7-days animals (201 blocks), four 30-days animals (63 blocks), six 60-days animals (102 blocks), and seven 180-days animals (103 blocks) were prepared. Histological sections were cut at 5-µm intervals from the paraffin blocks on a rotary microtome and mounted on charged glass slides, stained with hematoxylin and eosin, and modified Movat Pentachrome stains.

Histological Assessment

Histological assessment of the renal vessels, adjacent nerve fascicles, arteries, and surrounding soft tissue was performed by light microscopy, and ordinal data were collected for multiple parameters including endothelial cell loss, arterial and venous medial injury (depth and circumference), inflammation, soft tissue injury and necrosis, and arteriolar injury. These parameters were semiquantified using a scoring system of 0 to 4: 0=none; 1=minimal, 2=mild; 3=moderate; and 4=severe. Maximum nerve damage was assessed based on the extent of nerve injury observed histologically. Perineural injury including perineural inflammation or fibrosis and endoneurial injury including vacuolization, digestion chambers, pyknotic nuclei, and necrosis were evaluated. Nerve regeneration refers to the disordered proliferation of new nerves consisting of regenerating clusters of axons surrounded by Schwann cells forming an onion bulb with and without macrophage infiltration within an area of fibrosis.

Endothelium damage was assessed circumferentially as 0=no endothelial loss, 1=endothelial loss <25% of vessel circumference, 2=endothelial loss 25% to 50% of vessel circumference, 3=endothelial loss 51% to 75% of vessel circumference, and 4=endothelial loss >75% of vessel circumference. Medial injury was evaluated by the depth and circumferential involvement separately: 0=no media change; grade 1=medial injury involving <25% of media depth/ circumference; grade 2=medial injury, 25% to 50% of media depth/ circumference; grade 3=medial injury 51% to 75% of medial depth/ circumference, and grade 4=medial injury >75% of medial depth/circumference. In addition, the presence of arterial medial thinning

Table 1. Number of Animals and Treated Renal Arteries in Each Time Point

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Number of Animals</th>
<th>Number of Treated Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>30 days</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>60 days</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>180 days</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>49</td>
</tr>
</tbody>
</table>

*Each 180-day animal received ipsilateral treatment.
was defined as thickness of media at the site of damage (mm)/unaffected media thickness (mm) <0.5 and was assessed as absent (0) or present (1). These changes were the result of severe smooth muscle cell loss/necrosis within the media, accompanied by thinning. Arteriolar injury was evaluated using a grading system of 0 to 4, which was applied with respect to inflammation/fibroinoid necrosis: 0=none, 1=minimal, 2=mild, 3=moderate, and 4=severe. Prevalence of denatured collagen, which seems as basophilic staining of collagenous tissue from thermal injury under hematoxylin and eosin stain, was assessed as absent (0) or present (1). To evaluate the extent of tissue damage in a circumferential dimension, the following parameters were assessed after dividing the entire vascular and peri-vascular circumference into 4 equal quadrants: (1) number of quadrants with nerve fascicles; (2) number of quadrants with injured nerve fascicles (grade ≥2); (3) number of quadrants with moderate to severely injured nerve fascicles (grade ≥3); and (4) number of quadrants with injured peri-arterial soft tissue. Digital images from Movat pentachrome stained sections were acquired at ×1.25 magnification. Distance from arterial lumen to deepest tissue damage in each section was measured using a National Institute of Standards and Technology traceable calibrated microscopic system with morphometric software (IP Laboratory for Mac OS X, Scanalytics, Rockville, MD).12

### Immunohistochemical Staining of Peri-Vascular Nerves and Scoring Criteria

Immunohistochemical staining was performed on selected sections after sectioning of the paraffin blocks. Three representative renal vessels were selected from each time point (7, 30, 60, and 180 days) after histopathologic scoring was completed. From each vessel, a total of three sections (proximal to radiofrequency ablation site, maximum radiofrequency ablation injury site and distal to radiofrequency ablation site) were selected for staining and analysis. The extent of nerve injury on the stromal elements was assessed after immunostaining against S-100 protein (dilution 1: 4000; Dako, Carpinteria, CA). Presence of functionally intact sympathetic axons within the nerve fascicles was assessed after immunostaining against tyrosine hydroxylase (TH) (1: 100; EMD Millipore, Billerica, MA), a functional indicator for norepinephrine synthesis. The intensity and distribution of staining were scored semiquantitatively using a scoring system of 0 to 3: (0)=no reaction (strong functional damage); 1=patchy/very weak reaction (moderate functional damage); 2=weak reaction (mild functional damage); and 3=strong reaction (no functional damage).12

### Statistical Methods

After scoring in all sections, the maximum score as well as the mean score in each vessel were calculated. Maximum scores were used for statistical analysis because mean scores included non-treated segments. The results of mean scores were described in the Table I in the Data Supplement. Results were expressed as mean±SD. Normality of distribution was tested with the Wilk–Shapiro test. Statistical comparisons were performed by linear generalized estimating equation modeling with an assumed Gaussian distribution, an identity link function (normally distributed data) or a log link function (skewed data), and an assumed exchangeable structure for the within-cluster correlation matrix in consideration of the clustered nature of ≥1 individual measurements from 1 animal. Skewed data after log transformation were analyzed with the Kruskal–Wallis test. For the comparison of immunohistochemistry (1 individual measurement from 1 animal), comparisons of variables with normal distribution were accomplished by 1-way ANOVA, whereas comparisons of variables with skewed data distribution were performed by the Kruskal–Wallis test. A value of P<0.05 was considered statistically significant. All analyses were performed with the SPSS software (version 19; Chicago, IL) and JMP 5 (SAS Institute, Cary, NC).

### Results

#### Chronological Comparisons of Study Cohorts

A total of 469 sections were examined from 28 animals with 56 renal arteries. Sections from untreated controls (n=49) and incomplete sections (n=22) were excluded from main analysis. Therefore, a total of 398 sections were used for the analysis. A total of 49 renal arteries, from 28 animals, were compared according to the time points after radiofrequency ablation. Semicuantitative scoring and measurements of maximum depth were performed blinded by CVPath (K. S. scored all sections and confirmed by R. V.) without knowledge of the time of euthanasia until tabulation and statistical analysis. After the statistical analysis of the hematoxylin and eosin and Movat stained sections, additional 5-µm sections were cut and stained by immunohistochemical methods for presence of TH and scored for the intensity of staining per treatment group.

Tables 2 and 3 and Figure 1 show the comparison of nerve injury between groups. Nerve injury score was significantly

### Table 2. Comparison of Nerve Injury Between Groups, Evaluated by Standard Histopathology

<table>
<thead>
<tr>
<th>Nerve Injury Evaluated by Standard Histopathology</th>
<th>7 Days (n=22)</th>
<th>30 Days (n=8)</th>
<th>60 Days (n=12)</th>
<th>180 Days (n=7)</th>
<th>Overall P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injured nerve (score ≥2) quadrants/nerve quadrants: maximum/artery</td>
<td>1.0±0.1*</td>
<td>0.9±0.1</td>
<td>0.9±0.2</td>
<td>1.0±0.6</td>
<td>0.019</td>
</tr>
<tr>
<td>Injured nerve (score ≥3) quadrants/nerve quadrants: maximum/artery</td>
<td>0.7±0.3†</td>
<td>0.3±0.4</td>
<td>0.3±0.3</td>
<td>0.4±0.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. Generalized estimating equation model was used.

*P<0.05 for 7 days vs 60 days, †P<0.05 for 7 days vs 30 days.

### Table 3. Comparison of Nerve Injury Between Groups, Evaluated by Immunohistochemistry

<table>
<thead>
<tr>
<th>Functional Nerve Injury Evaluated by Immunohistochemistry</th>
<th>7 Days (n=3)</th>
<th>30 Days (n=3)</th>
<th>60 Days (n=3)</th>
<th>180 Days (n=3)</th>
<th>Overall P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH score (0–3) proximal to RF ablation site*</td>
<td>2.3±0.6</td>
<td>2.3±0.6</td>
<td>2.7±0.6</td>
<td>3.0±0.0</td>
<td>0.33</td>
</tr>
<tr>
<td>TH score (0–3) within RF ablation site*</td>
<td>1±1</td>
<td>0.7±0.6</td>
<td>2.7±0.6</td>
<td>2.7±0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>TH score (0–3) distal to RF ablation site*</td>
<td>1.7±1.2</td>
<td>1.7±1.2</td>
<td>2.3±0.6</td>
<td>2.7±0.6</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. Kruskal–Wallis test, or 1-way ANOVA test was used. RF indicates radiofrequency; and TH, tyrosine hydroxylase.

*All nerves except 1 section (score 1) showed score 3 with S-100 antibody.
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**Chronologic Changes Following RF-RDN**

greater at 7 days compared with 30, 60, and 180 days ($P<0.001$).

Injured nerve score of ≥3 in number of quadrants/total quadrants identified as having nerves were also significantly higher at 7 days compared with 30, 60, and 180 days ($P<0.001$).

Representative images of the progression of renal nerve injury are shown in Figure 1, and representative images of injured nerves after immunostaining are shown in Figure 2. At 7 days, the affected nerves show coagulation necrosis with digestion chambers, vacuolization, nuclear pyknosis, and significant loss in nuclear and cellular detail. Affected nerve bundles also seem moderately to markedly swollen. The nerve and the surrounding connective tissue are frequently infiltrated by mononuclear inflammation. At 30 days, the affected nerves show moderate perineural fibrosis. The nerve capsule is thickened because of the fibrotic response and contains mononuclear inflammatory cells, as well as prominent capillary neovascularization. The nerve parenchyma is hypercellular with haphazardly arranged and mild vacuolated Schwann cells and scattered mononuclear inflammatory cells. At 60 days after treatment, the nerve lesions are predominated by...
maturing perineural and capsular fibrosis with capillary neo-
vascularization that extends throughout the nerve parenchyma.
Inflammation is greatly reduced to scant individual mononu-
clear cells. The nerve lesions at day 180 are characterized by a
further maturing fibrotic nerve capsule and shrunken nerve
bundles. Inflammation is absent. Capsular and parenchymal
capillaries are prominent. The Schwann cells are more sparsely
positioned yet still forming sheets and smaller fascicles.

TH staining score within the radiofrequency ablation zone
was significantly less at the 7 and 30 days compared with 60
and 180 days (P=0.01). Focal nerve regeneration was generally
observed only within the radiofrequency ablation site in 2 of 12
renal arteries (17%) at 60 days and 5 of 7 animals (71%) at the
180 days. There was no nerve regeneration observed distally to
any of the radiofrequency lesions. The nerve regeneration was
characterized by disordered proliferations of multiple small
nerves consisting of regenerating clusters of axons surrounded
by Schwann cells forming an onion bulb with or without macro-
phage infiltration within an area of fibrosis. Representative
images of nerve regeneration are shown in Figure 3.

Table 4 and Figure 4 show comparison of arterial and peri-
arterial tissue injury between groups. Renal arterial medial
depth injury was not significantly different between groups while
arterial medial thinning score was significantly greater at the
7days mark compared with the 30, 60, and 180 days. Renal arterial medial circumferential injury (%) was greatest at 7 days, followed by 30 and 60 days, and the least injury was seen at 180 days (P=0.046). Arterial medial damage was observed at 7 days and was characterized by acute smooth muscle cell loss, which resulted in media thinning. Media thinning was mostly observed at the 7-day mark and was rarely seen beyond ≥30 days. However, proteoglycan replacement of smooth muscle cell loss was frequently seen at 30 days and beyond. Representative images of the extent of
arterial injury between groups are shown in Figure 4. Injury of
the veins was minimal in all groups. Arteriolar injury score was significantly greater at 7 days compared with 30, 60, and 180 days (P=0.0001). Arteriolar damage at 7 days was characterized by acute fibrinoid necrosis of the media and endothelium with smooth muscle cell loss, whereas at 30 days and beyond it was characterized by the presence of smooth muscle cells and adventitial fibrosis. The number of quadrants of soft tissue injury, which was characterized by the presence of denatured collagen, fat necrosis, and granulation
tissue, was greatest at 7 days, followed by 30 and 60 days and
was the least at 180 days (P<0.001). Denatured collagen was
predominantly observed at 7 days (P<0.001). Distance from the intima to the deepest site of tissue injury was greatest at 7 days, followed by the 30, 60, and 180 days without
reaching statistical significance.

Discussion
The present histopathologic study assessed chronologically
histopathologic changes after radiofrequency ablation in a
time course between 7 and 180 days. The most salient find-
ings are listed below:

1. Radiofrequency-induced nerve injury was characterized
acutely (7 days) by nerve necrosis (coagulation necrosis with
total or focal absence of nuclei within nerve bundles) and
degeneration characterized by digestion chambers, vacuolization,
pyknotic nuclei, and inflammation and chronically (60 and 180
days) by perineural fibrosis and nerve atrophy.

2. Acute nerve injury peaked at 7 days after radiofrequency abla-
tion procedure and gradually declined thereafter ≤180 days.

3. Functional nerve damage assessed by the attenuated staining
patterns for TH also peaked at 7 days but was sustained at 30
days, with partial recovery at later time points.

Table 4. Comparison of Maximum Arterial and Peri-Arterial Tissue Injury Between Groups

<table>
<thead>
<tr>
<th></th>
<th>7 Days (n=22)</th>
<th>30 Days (n=8)</th>
<th>60 Days (n=12)</th>
<th>180 Days (n=7)</th>
<th>Overall P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal artery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial loss</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>NA</td>
</tr>
<tr>
<td>Media thinning</td>
<td>1.0±0.2†‡</td>
<td>0.1±0.4</td>
<td>0.1±0.3</td>
<td>0±0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Media injury</td>
<td>4±0</td>
<td>3.9±0.4</td>
<td>3.9±0.3</td>
<td>3.1±1.6</td>
<td>0.225</td>
</tr>
<tr>
<td>Media injury</td>
<td>2±0.6‡</td>
<td>1.9±0.4§</td>
<td>1.7±0.7</td>
<td>1.3±0.8</td>
<td>0.202</td>
</tr>
<tr>
<td>Arterioles</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>NA</td>
</tr>
<tr>
<td>Soft tissue injury</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injured soft tissue</td>
<td>4±0†‡</td>
<td>1.6±1.1</td>
<td>2.2±1.0</td>
<td>1.4±1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Denatured collagen</td>
<td>2.0±0.4†‡</td>
<td>1.4±0.5</td>
<td>1.5±0.5</td>
<td>1.0±0.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Distance to deepest injury (mm):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maximum/artery</td>
<td>7.3±2.4</td>
<td>6.1±3.5</td>
<td>6.1±3.1</td>
<td>6.2±3.3</td>
<td>0.660</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. Generalized estimating equation model or Kruskal–Wallis test was used.

*P<0.05 for 7 days vs 30 days, †P<0.05 for 7 days vs 60 days, ‡P<0.05 for 7 days vs 180 days, §P<0.05 for 30 days vs 180 days.
4. Focal nerve regeneration was observed only at the sites of radiofrequency lesions in 17% of arteries at 60 days and in 71% of arteries after 180 days.

5. Radiofrequency-induced injury of vascular and peri-vascular soft tissue also peaked at 7 days; the arterial media showed medial smooth muscle loss and necrosis with proteoglycan-collagen replacement over time ≤180 days.

Although radiofrequency RDN is clinically approved in Europe and other countries, preclinical studies using radiofrequency catheters remain sparse. Steigerwald et al.11 investigated the histopathology of peri-renal nerves and renal artery after radiofrequency RDN in the swine model using the Symplicity RDN system. The time points of follow-up was either hyperacute 45 minutes or subacute, 10 days. Although local loss of the endothelium was observed in the hyperacute phase, arterial media injury and nerve degeneration such as vacuolization and perineural fibrosis were only observed in the subacute phase.11 Rippy et al.10 only investigated the histopathologic findings after radiofrequency RDN at 6 months (chronic phase) in the swine model using the Symplicity RDN system. Renal nerve injury was characterized as healing fibrosis causing a thickening of the surrounding perineurium in affected nerves.10 Renal arterial injury was characterized by fibrosis involving 10% to 25% of the media.10 Despite the undoubted relevance of these preclinical investigations, a systematic and chronological investigation of histopathologic changes after RDN has not been described to date and may lead to a greater understanding of this novel technology. In the current study, we not only examined acute and chronic histopathologic responses but also characterized those occurring during the transition phase (30 and 60 days) of vascular healing after RDN with emphasis on the type of nerve changes and the depth of soft tissue injury. In this regard, the present study provides biological insights from multiple time points applying quantitative evaluation measures, which enabled us to draw parallels between the histopathologic changes that involve nerves and arterial/peri-arterial soft tissue in a time span ranging from subacute to transitional period, with extension to late chronic phase.

Relevance of Morphological Versus Functional Assessment

Although morphologically greatest injury was observed at 7 days and substantially declined thereafter, functional damage evaluated by TH staining peaked at 7 days and was sustained until 30 days. However, increased staining was noted after 60 and 180 days, which suggests that axonal restoration may occur after radiofrequency denervation, and is also explained by the resolution of injury such as edema with eventual fibrosis. Our findings also confirm that nerve regeneration occurs which is demonstrated by S100 and TH positivity by immunohistochemical staining. However, the pathophysiological relevance of these morphological findings must be confirmed in future studies by investigating the extent of norepinephrine levels in the kidney over time. Nerve regeneration is a healing process of damaged peripheral nerves13 and is likely to be influenced by time, species, and age. Although the timing and duration of preclinical studies can be controlled, the influence...
of species is likely to have the greatest impact on nerve regeneration: weeks in rats, months in dogs, and months to year(s) in humans.14–17

Clinical Implications
Short-term safety aspects of RDN procedures mainly refer to the absence of adverse vascular effects in renal arteries as radiofrequency energy is delivered transmurally.18 It is not surprising that there was absence of arterial endothelial damage at 7 days after treatment in the current study because re-endothelialization has previously been reported to be complete within 10 days in other preclinical RDN studies.11 Although recent optical coherence tomography imaging studies observed occasional small nonocclusive mural thrombi in the renal arteries after RDN procedures,19 it is clear from the HTN-3 study9 that the RDN procedure was safe. On a morphological level, the current study showed that nerve regeneration was observed within the region of radiofrequency ablation and yet, complete neuronal restoration was absent at 180 days. Therefore, sustained antihypertensive treatment effect, which has been reported beyond this time point, is not surprising.

Arterial media thinning was observed at 7 days, which reflects direct injury (necrosis) by radiofrequency energy. Proteoglycan replacement of smooth muscle cell loss occurs subsequently after necrosis and is the initial healing process of the injured media. Of note is that the medial circumferential injury was least at 180 days, which suggests some potential of the media to regenerate or shrink in the chronic phase because of type 1 collagen cross-linking. These results support the safety of the Symplicity system.

Recent results of Symplicity HTN-3, which failed the primary efficacy end point at 6 months, underscore the importance of systematic preclinical studies to better understand the mechanism of RDN.9 Currently, no biomarker has been identified that is consistently and reliably elevated or suppressed during RDN of human sympathetic nerves. Therefore, it is difficult to clinically confirm at the time of RDN whether sympathetic nerves are denervated during and after the procedure. At this time, only preclinical histopathologic studies allow us to precisely evaluate histologically the ablation characteristics by different devices. The knowledge of human renal anatomy20 as well as the ablation geometry by each device is of utmost importance for the determination of effective ablation strategy.

Study Limitations
First, the findings of this swine study should be interpreted with caution because the influence of underlying atherosclerosis in human renal arteries cannot be assessed from a healthy animal model. Nevertheless, renovascular anatomy in swine is similar to human, whereas peri-renal nerve distribution in swine is different from that in human.12,20,21 Also, the life span of swine is dramatically shorter compared with man,22 and therefore the time course of nerve and vascular regeneration may be substantially prolonged in humans compared with swine. Although a portion of the samples from the 180 days animals were published previously by Rippy et al,10 scoring in our study was performed independently in a blinded manner without knowledge of previous results. However, we did not correlate our histological findings to the extent of norepinephrine levels in the kidney or did we study the RDN effects in a hypertensive animal model. Finally, our study evaluated histopathology after radiofrequency-RDN in a healthy porcine model. Therefore, it remains unknown whether chronological changes of nerve injury and regeneration may be different if other sources such as cryo or ultrasound energy are used.23,24

Conclusions
Nerve injury after radiofrequency RDN is greatest in the subacute phase (7 days) compared with transitional phase (30 and 60 days) and chronic phase (180 days), with peak functional nerve damage seen at 30 days. Focal nerve regeneration similar to amputation neuromas was only observed at the sites of radiofrequency injury in the transitional phase (60 days) and was sustaining into the chronic phase (180 days). Renal arterial injury is greatest in the subacute phase (7 days) and least in the chronic phase (180 days), suggesting complete healing of arterial wall and soft tissue at this time point in the swine model.

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Disclosures
Dr Sakakura has received speaking honorarium from Abbott Vascular, Boston Scientific, and Medtronic CardioVascular. Drs Tunev, O’Brien, and Melder are employee of Medtronic Cardiovascular. Dr Joner is a consultant for Biotronik and Cardionovum and has received speaking honorarium from Abbott Vascular, Biotronik, Cordis Johnson&Johnson, Medtronic, and St. Jude. Dr Virmani receives research support from 480 Biomedical, Abbott Vascular, Atrium, Biosensors International, Biotronik, Boston Scientific, Cordis Johnson & Johnson, GSK, Kona, Medtronic, Micropor Medical, OrbusNeich Medical, ReCor, SINO Medical Technology, Terumo Corporation, and W.L. Gore; has speaking engagements with Merck; receives honoraria from 480 Biomedical, Abbott Vascular, Biosensors International, Boston Scientific, CeloNova BioSciences, Claret Medical, Cordis Johnson & Johnson, Ketuxis Bard, Medtronic, Terumo Corporation, and W.L. Gore; and is a consultant to 480 Biomedical, Abbott Vascular, Medtronic, and W.L. Gore. The other authors report no conflicts.

References

5. Sakakura et al Chronologic Changes Following RF-RDN

6. Sakakura et al Chronologic Changes Following RF-RDN
Renal sympathetic denervation in patients with treatment-resistant hypertension (the SYMPLICITY HTN-2 trial): a randomised controlled trial. 


Sakakura et al  Chronicologic Changes Following RF-RDN


Comparison of Histopathologic Analysis Following Renal Sympathetic Denervation Over Multiple Time Points
Kenichi Sakakura, Stefan Tunev, Kazuyuki Yahagi, Amanda J. O'Brien, Elena Ladich, Frank D. Kolodgie, Robert J. Melder, Michael Joner and Renu Virmani

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Supplemental Material

Supplemental Table 1. Comparison of nerve, arterial, and peri-arterial tissue injuries between groups

<table>
<thead>
<tr>
<th>Injury evaluated by standard histopathology</th>
<th>7-days (n=22)</th>
<th>30-days (n=8)</th>
<th>60-days (n=12)</th>
<th>180-days (n=7)</th>
<th>Overall P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve injury (score 0-4): mean/artery</td>
<td>2.6±0.7†‡¶</td>
<td>1.8±0.4</td>
<td>1.7±0.3</td>
<td>1.7±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Injured nerve (score ≥2) quadrants/nerve quadrants: mean/artery</td>
<td>0.7±0.2</td>
<td>0.6±0.2</td>
<td>0.5±0.2</td>
<td>0.6±0.3</td>
<td>0.10</td>
</tr>
<tr>
<td>Injured nerve (score ≥3) quadrants/nerve quadrants: mean/artery</td>
<td>0.3±0.2†‡¶</td>
<td>0.06±0.1</td>
<td>0.1±0.1</td>
<td>0.1±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endothelial loss (score 0-4): mean/artery</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>NA</td>
</tr>
<tr>
<td>Media thinning (score 0-1): mean/artery</td>
<td>0.3±0.1†‡¶</td>
<td>0.03±0.1</td>
<td>0.01±0.04</td>
<td>0±0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Media injury depth (score 0-4): mean/artery</td>
<td>1.7±0.4</td>
<td>1.7±0.7</td>
<td>1.5±0.7</td>
<td>1.1±0.9</td>
<td>0.239</td>
</tr>
<tr>
<td>Media injury circumferential (score 0-4): mean/artery</td>
<td>0.8±0.3¶</td>
<td>0.9±0.3¶</td>
<td>0.6±0.3</td>
<td>0.4±0.3</td>
<td>0.005</td>
</tr>
<tr>
<td>Media injury circumferential (%): mean/artery</td>
<td>14±5¶</td>
<td>13±5¶</td>
<td>10±6</td>
<td>7±6</td>
<td>0.013</td>
</tr>
<tr>
<td>Arterioles injury (score0-4): mean/artery</td>
<td>2.4±0.8†‡¶</td>
<td>0.6±0.5</td>
<td>0.8±0.4</td>
<td>0.7±0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Injured soft tissue (number of quadrants): mean/artery</td>
<td>1.3±0.4†‡¶</td>
<td>0.8±0.3</td>
<td>0.8±0.3</td>
<td>0.5±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Denatured collagen (score0-1): mean/artery</td>
<td>0.4±0.1†‡¶</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Distance to deepest injury (mm): mean/artery</td>
<td>4.9±1.7†¶</td>
<td>3.8±1.2</td>
<td>3.7±1.7</td>
<td>3.5±1.0</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD.

†=P<0.05 for 7-days vs. 30-days, ‡=P<0.05 for 7-days vs. 60-days, ¶=P<0.05 for 7-days vs. 180-days.

Generalized estimating equation model, Kruskal-Wallis test, or 1-way ANOVA test was used.