Sub-acute Safety and Efficacy Evaluation of A Single versus Double Treatment Cycles of a Monopolar Radiofrequency Catheter-Based Renal Nerve Ablation and its Chronic Evolution in a Large Animal Model

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Abstract

We aimed to evaluate the transcatheter renal denervation (RDN) effects delivered by a mono-electrode catheter in a large animal model including safety implications of delivery of one cycle versus two cycles of ablations.

Methods & Results

18 animals underwent bilateral RDN; 4 untreated naïve swine were enrolled as controls for norepinephrine levels (NE) only. Animals received 120-second (follow up-7.30 and 90 days) or 240-second cycles ablations (follow up-7 days). Norepinephrine evaluation, histology and immunohistochemistry evaluation was performed. No luminal obstruction was observed at follow up. A 70% decrease in NE levels (76.68±57.87ng/g) was observed at 7 days, 81% at 30 days (49.05±45.81ng/g), and 51% at 90 days (12.3±73.2 ng/g) compared to naïve controls (254.1±54.1ng/g; p<0.001). Histologically, the thermal effect extended to a complete circumferential involvement with a depth ~8mm. The primary histological feature at 7 days was nerve necrosis and distal atrophy; at 30 days, necrosis was replaced by healing changes of fibrosis. Neuromatous regeneration was apparent at 30 days at RF treated levels. At 90 days these features progressed to become more conspicuous. There were no appreciable differences in depth and circumferential extent of RF injury between one and two cycle treatment groups.

Conclusion

RDN performed with a mono-electrode catheter (Iberis) appears to be safe following single or double-cycle RF ablation. NE decrease following RDN was demonstrated at 7, 30, and 90 days compared to naïve controls, suggesting efficient nerve ablation with the device as intended for human use.

Keywords: Renal Denervation, Porcine Animal Model, Terumo Iberis™ System, Safety Preclinical Evaluation

Citation:

Introduction

Over-activation of the sympathetic nervous system (SNS) has been definitively identified as one of several known factors contributing to the development and progression of hypertension.1 Transcatheter radiofrequency ablation of the renal nerves has been proposed as a therapeutic option for patients with uncontrolled hypertension.2 The safety of radiofrequency ablation of renal nerves has been demonstrated in several clinical
trials of various devices that deliver RF energy through the wall of the renal artery. However, the efficacy of this therapy were brought into question in light of the results of the Symplicity HTN-3 trial.\(^2\) Compared to a sham control, there was no statistical difference in office blood pressure or 24-hour ambulatory blood pressure monitoring at 63 and 12 months.\(^4\) Debate continues regarding the reasons for failure of the trial, including limited operator experience with proper denervation 5–10. In contrast, the DENER-HTN study recently reported superiority of renal denervation over medical treatment alone in reducing ambulatory blood pressure.\(^1\) Further clinical and preclinical trials of renal denervation are being conducted with hope that further light can be shed on how renal denervation can be utilized to benefit various pathophysiologic states.

In this preclinical safety and efficacy study, we aimed to evaluate the effects of radiofrequency treatment (RF) delivered by a monopolar electrode catheter (Iberis™, Terumo) in a large animal model including safety implications of delivery of one cycle versus two cycles of RF ablations.

**Methods**

All procedures were in compliance with the USDA Regulations and the Animal Welfare Act (9 CFR Parts 1, 2 and 3), following the Guide for the Care and Use of Laboratory Animals. The protocol for these studies was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of T3 Laboratories, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

**Device Description**

The Iberis™ Renal Denervation System (CE Marked)\(^2,13\) is a highly flexible 6F guide catheter compatible device with single point of contact energy delivery capabilities. Iberis™ is designed to deliver low level RF energy (8 watts) through the wall of the renal artery to target the renal sympathetic nerves. The system consists of two major components: a sterile single use ablation catheter with an electrode tip and a proprietary generator, which delivers RF energy sufficient for ablating sympathetic nerves adjacent to the treatment site. The RF generator displays temperature, impedance, radiofrequency power, procedure countdown and warning/instructional message screens (Figure 1).

**Animal Model and Interventional Procedure**

Juvenile female domestic Yorkshire swine (body weight 51–62 kg) were included in this study; 18 animals underwent renal artery denervation procedure and 4 animals served as naïve controls for renal cortical tissue harvest only. Animals were maintained on a standard chow diet and were fasted at least 12 hours before the interventional or renal cortical tissue harvest procedure. On the day of treatment, one intramuscular (IM) muscarinic anticholinergic dose of atropine 0.04 mg/kg was given before the procedure. Induction of anesthesia was achieved with a rapid-acting dissociative anesthetic. All animals underwent endotracheal intubation and were maintained with a continuous inhalation of 0.5–5% Isoflurane in oxygen. Femoral arterial access was obtained under general anesthesia using a percutaneous Seldinger technique. All animals received 325 mg of Aspirin single dose daily for a minimum of one day before the initial procedure. This same dose was administered on the day of the procedure and daily until the completion of the study. Anticoagulation with heparin was achieved during the procedure (50–75 units/Kg IV) to maintain an activated clotting time ≥250 seconds. After baseline angiography of the renal arteries, catheter-based sympathetic denervation was performed bilaterally, within the main stem of the renal artery at four to six evenly distributed sites in the arteries ensuring that the whole length of the artery from the bifurcation to the ostium of the renal artery was covered. No renal accessory arteries were observed in the evaluated swine. RF power was set at 8 watts for all ablations. RF treatment was applied from the first renal artery bifurcation with subsequent ablations performed in a retrograde fashion ~5 mm apart after repositioning of the catheter in order to maximize the number if treatments toward the renal artery ostium. Pigs were randomized to receive 1 treatment cycle of 120 seconds (n=24) of energy delivery at each ablation site. These arteries were evaluated at 7, 30 and 90 days. Eight renal arteries received 2 back-to-back treatment cycles (240 seconds total) of energy delivery at each ablation site. Arteries that received 2 treatment cycles were evaluated for 7 days only. Renal arteries receiving no RF treatment were deemed “naïve” (n=3) and served as a negative control. Nitroglycerin 200mcg was injected directly into renal arteries prior to ablation to prevent vasospasm. Following post-treatment final angiography, Isoflurane was discontinued and the animal was exsanguinated. Buprenorphine 0.005 mg/kg IM was injected for routine pain management. Animals received Cefazolin as prophylaxis (1 g IM). At the terminal time point (7, 30 or 90 days), animals were heparinized (200U/kg IV) and following final renal angiography and prior to euthanasia, a midline laparotomy was performed and fresh renal cortical tissues harvested for norepinephrine analysis (Figure 2). Animals that received 2 treatment cycles were not subjected to cortical tissue harvest procedures (Figure 2).

**Tissue Collection Bioanalytical Analysis and Histopathology**

Gross visual examination of the urinary bladder, intestines and other tissues surrounding the renal arteries and kidneys was performed. The renal capsule was dissected to expose the kidney. To obtain tissues specimens for norepinephrine analysis, a superficial section of the dorsal and ventral aspect of the renal cortex was removed such that the tissue samples were approximately 2–3 mm thick. Each tissue sample (dorsal and ventral) was divided into a caudal, mid, and cranial section (for a total of 6 sections per kidney). Excess blood was blotted from the tissue samples, and each sample section was weighed. Samples were trimmed as necessary to obtain a weight of approximately 1.0 g. Each sample section was then placed in a corresponding pre-labeled vial (each containing 4.0ml of aqueous 10mg/ml sodium metabisulfite solution) and submerged. Vials were immediately transferred into liquid nitrogen to flash freeze, then once fully frozen, transferred into a -80°C freezer for storage prior to norepinephrine concentration determination. Once samples for norepinephrine analysis were obtained, the animal was euthanized via IV bolus of KCL while under deep inhalant anesthesia. At necropsy, the abdominal aorta was cannulated
and gravity flushed with physiologic saline solution to clear blood from the renal vasculature. When the vessels were clear of blood the cannulated tissues were gravity perfusion fixed with 10% neutral buffered formalin (NBF) solution. The renal arteries were pressure perfused for 5 minutes at a height of ~140-165 cm above tissues being perfused to provide ~100 mmHg of pressure. The treatment area and surrounding tissues were extracted en-bloc, to include the following tissues: abdominal aorta; renal arteries; kidneys and surrounding tissue (retroperitoneal connective tissue, renal capsule, perirenal fascia, and peritoneum), underlying posterior psoas and sublumbar muscles, adrenal glands and ureters (Figure 2). Tissue explants were immersed into 10% NBF while supported by a solid cork backing to ensure stability and structural integrity of the tissue block.

Tissue Preparation for Histology and Immunohistochemical Analysis

After no less than 24 hours of 10% NBF fixation, the aorta was bisected to expose the renal ostia. The renal artery and approximately 1.5–2 cm radius of the surrounding retroperitoneal connective tissues and fasciae were isolated by removing excess tissue to create a roughly 3-cm-wide renal “stump” centered on the renal artery (Figure 2b). Equidistant sections were taken from the ostium to the hilus of the renal stump, resulting in 8 to 13 cross-sections along the length of the treated renal artery that included associated soft tissues (lymph nodes, soft connective tissue, veins, and nerves) and underlying skeletal muscle. The ureters and adrenal glands were also included when present within the block of tissue presented for sectioning. The resulting tissue slices were dehydrated in ascending concentrations of alcohol and embedded in paraffin (Figure 2c, 2d). Serial 5 µm sections were stained with hematoxylin and eosin (H&E) and Elastic Trichrome (ET) for each time point (7, 30 and 90 days).

The renal arteries were assessed, for endothelial coverage, thrombosis, neointima formation, procedural vascular wall injury, media hypocellularity, nerve atrophy, perineurial/endoneurial fibrosis, and neuromatous regeneration and inflammation by a pathologist who was blinded to the randomization and therapy delivered for. These histopathology features were evaluated under a semiquantitative score from ‘0’ (histological feature not present) to ‘3’ (overwhelmingly presence of histological feature), except for endothelialization, which score was from ‘0’ (~<25% of the luminal surface covered by endothelium) to ‘4’ (~>95% of the luminal surface covered by endothelium). Histological findings involving the nerves were scored on a 0-3 scale according to the proportion of nerves displaying the feature in a given section, taking into account the number and the size of respective nerves (score 1 = change present, but minimal feature limited to one or a few small nerves; score 2 = Notable feature involving several nerves or at least one larger bundle, score 3 = Overwhelming feature involving most nerves within the section). Samples of kidneys were assessed microscopically for any pathological changes or downstream effects to include the presence of thrombi or emboli, inflammation, granulomas, necrosis, renal infarcts, or any other microscopic changes. Quantitative assessments were made using an ocular micrometer, including measurements of the depth of RF changes and media thickness at and away from the RF contact point, and overall circumferential extension of the RF lesion (estimated visually) in and around the treated artery.

The methods used for the immunohistochemistry have been previously described. Briefly, 2 representative sections were selected for evaluation by immunohistochemistry of expression of Schwann cells (S100), tyrosine hydroxylase (TH) and Growth Associated Protein 43 (GAP43). Following antigen retrieval, 3% hydrogen peroxide and background Sniper were used for blocking endogenous peroxidase and protein block respectively (Biocare Medical, Concord, CA, USA). Slides were incubated with each primary antibody (rabbit polyclonal anti-S100 [Abcam, Cambridge, MA], rabbit polyclonal anti-TH [Millipore, Billerica, MA] and rabbit polyclonal anti-GAP43 [Novus Biologicals, Littleton, CO]), secondary antibody and stained and counterstained according to previously described protocols. The resulting slides were examined via light microscopy and were described based on the presence of positive nerve fibers (TH and GAP43) or Schwann cells (S100) in normal and affected nerves.
Kidney Norepinephrine Assay
Cortical tissue samples were kept frozen until analysis at the bioanalytical test site (Tandem Labs). The entire volume of the tissue samples was homogenized with 3/16” stainless steel ball bearings in a bead homogenizer. Samples were centrifuged and filtered. Calibration standards were prepared in aqueous 10 mg/mL sodium metabisulfite and the calibration range was 0.100 to 100 ng/mL. Stable isotope labeled norepinephrine was added to each sample (standards, QCs and study samples) as an internal standard. Samples were derivatized with aminoethyl diphenylborinate followed by solid phase extraction on an Agilent C18 Versaplate. Elution of the samples with 0.1 M perchloric acid removed the diphenylborinate group leaving free norepinephrine. Samples were then injected onto an HPLC-MS/MS system for quantitation. Separation was performed on a Waters UPLC BEH C18 (1.8µm, 2.1 x 50 mm) HPLC column. Detection was with an API-5000 triple quadrupole mass spectrometer (AB-Sciex) with an electrospray source operated in the positive ion mode. Quantitation used analyte/internal standard peak area ratios. Calibration curves were generated with a linear regression and 1/x2 weighting. QC and study sample concentrations were back-calculated from the calibration curve. Study sample concentrations were then normalized to the reported tissue mass (concentrations reported as nanogram norepinephrine per gram of tissue).

Statistical Analysis
The Pearson and Spearman correlation coefficient was calculated to assess the correlation between norepinephrine concentration and histopathological data: continuous data in Pearson and semiquantitative scores in Spearman. For comparison of NE concentration in each time point with that in the control group, the data were analyzed by Dunnett’s multiple-comparison test followed by confirmation of the homogeneity of variance tested by Bartlett’s test (p > 0.05). Data were analyzed with EXSUS software (Ver 7.7.1; Arm Systex Co., Ltd., Osaka, Japan) in combination with SAS (Ver. 9.2 TS2M3, SAS Institute Inc., Cary, NC, USA).

Results
There were no early deaths, clinical conditions requiring urgent veterinary care or unscheduled euthanasia among the study animals. All animals survived to their designated terminal end point. No in vivo clinical pathology (blood analysis) pointed to any significant clinical impairment in any subject at any time point. A total of 36 renal arteries were evaluated from 18 animals receiving either one (n=24) or two (n=8) treatment cycles of radiofrequency ablations. 3 arteries were left untreated to serve as negative controls.

Angiography
The main renal artery length between the aorta and the main arterial bifurcation was 28.0±7.5 mm on average. At baseline, the mean luminal diameter (MLD) in the main renal artery was 5.37±0.4 mm. The characteristic image previously described as “notches” following RF treatment delivery was evident in all treated arteries (Figure 3). There was no MLD difference between baseline (7 days, 5.2±0.4mm; 30 days, 5.3±0.3mm; 90 days, 5.8±0.5mm, p for all >0.05) and follow up (7 days, 5.2±0.5mm [Δ-0.1±0.4mm]; 30 days, 5.5±0.3mm [Δ-0.2±0.2mm]; 90 days, 5.8±0.5mm [Δ0±0.3mm], p for all >0.05) with no evidence of percent diameter of stenosis (%DS; 7 days, -1.1±7.5%; 30 days, -4.4±4.9%; 90 days, -0.1±5.5%; p for all >0.05). The notches observed following treatment delivery at baseline were completely resolved at follow up. There was no angiographic difference between the renal arteries that received one or two treatment cycles evidenced by an identical MLD at baseline (5.2±0.3mm) and follow up (5.2±0.5mm, [Δ0±0.3mm], p with no %DS (1.5±6.4%). All arteries displayed a TIMI-3 flow pre and post treatment delivery with no evidence of thrombus or intimal dissection.

Renal Cortical Norepinephrine Evaluation
All analytical runs met the acceptance criteria set for the calibration curve points, for the lower limit of quantitation and for the quality control samples. NE levels were completely similar for the quality control samples. NE levels were completely similar between cranial, mid and caudal kidney sections, as well as between kidney sections across time points. %DS for the renal arteries that received one or two treatment cycles evidenced by an identical MLD at baseline (5.2±0.3mm) and follow up (5.2±0.5mm, [Δ0±0.3mm], p = 0.05) with no evidence of percent diameter of stenosis (%DS; 7 days, -1.1±7.5%; 30 days, -4.4±4.9%; 90 days, -0.1±5.5%; p for all >0.05). The notches observed following treatment delivery at baseline were completely resolved at follow up. There was no angiographic difference between the renal arteries that received one or two treatment cycles evidenced by an identical MLD at baseline (5.2±0.3mm) and follow up (5.2±0.5mm, [Δ0±0.3mm], p = 0.05) with no %DS (1.5±6.4%). All arteries displayed a TIMI-3 flow pre and post treatment delivery with no evidence of thrombus or intimal dissection.

Figure 3. Representative Angiographic Images
Legend: Baseline angiography of the renal arteries showed a clear lumen with no evidence of stenosis. Immediately after the delivery of the radiofrequency treatment the previously characteristic arterial “notches” were observed along the length of the renal artery (red arrows). There was no angiographic difference between the delivery of one or two treatment cycles of radiofrequency energy. TIMI flow was score III in every case without evidence of any intimal dissection or flow disturbance in the renal artery. At follow up there was no evidence of vascular stenosis in any of the treated arteries. The observed “notches” were completely resolved without any evidence of filling defects. TIMI flow remain score III in all instances.

Figure 4. Renal Cortical Norepinephrine Evaluation.
Legend: * p value <0.001 compared to control naïve kidneys.
kidneys of the negative control group displayed, statistically, the highest level of NE (254.05±54.12 ng/g) compared to all treated time point groups (p<0.001). Following the delivery of RF, there was a significant decrease of 70% in NE levels (76.68±57.87 ng/g, p<0.001 compared to control) 7 days post RDN. There was a significant decrease of 81% significant decrease in the levels of NE observed 30 days following RDN (49.05±45.81 ng/g, p<0.001 compared to control). Compared to control, at 90 days there was a 51% decrease in NE levels (123.66±73.19 ng/g, [p<0.001 compared to control] Figure 4).

Gross and Histological Evaluation

There was no evidence of collateral RF damage to any abdominal viscera in the one or two cycle exposed animals. At gross evaluation, there were no abnormal changes noted in any of the renal arteries (Figure 5). There were no abnormal gross or microscopic pathological changes observed in the renal parenchyma of any kidneys downstream of RF-treated renal arteries.

One Radiofrequency Treatment Cycle

In general and at all time points, the location where RF treatment was delivered was evident in the exposed artery by the involvement of the media. The media changes were confined to a well delineated area. The histological features following one (b) or two (c) treatment cycles were similar with a hyalinized media (b and c, solid double headed arrow) and necrosis of the surrounding connective tissue (amphophilic to basophilc staining of denatured collagen)(b and c, clear double headed arrow).

Figure 5. Representative Histology.
Legend: Panel I displays representative images of the radiofrequency ablation at 7 (a, d), 30 (b), and 90 (c) days. The dotted lines delineate the area of involvement and extension of the thermal injury in the wall of the artery (A), surrounding connective tissue and anatomical structures (M, muscle; LN, Lymph Node; U, Ureter; N, Nerve). The extension of thermal changes observed following one treatment cycle (a, b and c) is indistinct from the extension observed following two treatment cycles (d). Panel II. A representative image of a normal renal nerve (a) at high magnification shows compact fascicles of plump Schwann cells (asterisk) containing inconspicuous nerve fibers surrounded by a thin fibrous perineurium (arrow). The perineurial fibrous tissue response harbors the “nerve sprouts” (arrowheads) observed at 30 (d) and 90 (e) days. This response is not yet evident at 7 days (b) without IHC stains. At 7 days, the salient nerve changes include endoneurium necrosis (b, asterisk), hemorrhage and inflammatory cell infiltration (b, arrow head) with hyaline denaturation (coagulation necrosis) of the surrounding connective tissue and collagen (b, arrow). At 30 (d) and 90 (e) days, the neuramatos regenerasi is easily identifiable with conventional stains as increased fibrous tissue response (d, e; blue dotted line) and nerve “sprouts” (d, e; arrows) disrupting what was the original anatomy of the nerve (d, e; black dotted line). At all-time points, the RF-exposed nerves show increased perineurial and endoneurial fibrosis (f, g, h). The connective tissue expands the perineurium (f, double headed arrows; g and h, arrows) well beyond the original profile of the treated nerve (dotted line). Nerves distal to the location of delivery of the RF therapy typically show histological evidence of atrophy at 7 (j) and 30 (l) days. At 90 days (m), distal nerve atrophy is less conspicuous, likely due to regeneration of Schwann cells. The delivery of two treatment cycles followed at 7 days (c, g, k) displayed similar histological features compared to one treatment cycle (b, f, j). Panel III shows a normal renal artery wall and media (a) displaying a normal arterial thickness (a, double arrow head). The histological features following one (b) or two (c) treatment cycles were similar with a hyalinized media (b and c, solid double headed arrow) and necrosis of the surrounding connective tissue (amphophilc to basophilc staining of denatured collagen)(b and c, clear double headed arrow).
At 7 days, the mean depth of RF changes observed was 3.6±0.8mm with a 40±13% of vascular circumferential extension involvement. There was no suggestion of compromise of the integrity of the treated artery. On average, medial thickness at the point of RF delivery was slightly decreased (193±43µm) compared to the corresponding unexposed and normal wall (351±108 µm). There was no appreciable inflammation, medial or adventitial fibrosis (0.0±0.0) at 7 days. Medial hyalinization was present (1.0±0.4) and associated with nearly complete re-endothelialization (3.9±0.2). There was no presence of thrombus (0.0±0.0). Nerve necrosis (0.7±0.3), sometimes associated with reactive perineurial fibrosis (0.5±0.3) and nerve inflammation (0.5±0.3), at the exposed levels were the primary histological features. Distal nerve atrophy (1.1±0.7) was the characteristic histological feature that suggested relatively effective denervation of these nerves more proximally.

Immunohistochemistry at 7 days demonstrated uneven TH staining that was generally decreased compared to the untreated control. In nerves directly affected by RF thermal exposure there was virtually no TH staining. In some cases strong TH staining was found in disorganized nerve sprouts within the thickened perineurium. Early regenerative axons were very strongly GAP-43 positive and showed weak S100 staining. These changes were indicative of early regenerative attempts at the sites of direct RF nerve damage. Atrophic nerves present at 7 days showed very sparse and weak staining for all three markers.
At 30 days, mean tissue depth of RF tissue damage was 3.3±1.1mm, with a 38±9% of vascular circumferential extension involvement. In contrast to 7 days, the medial layer showed normal thickness at the point of RF therapy delivery (359±71 µm) similar to the unexposed wall (360±85 µm). There was very minimal inflammation recorded (0.1±0.3). Overt necrosis was no longer substantial and was replaced by healing changes of fibrosis. Compared to 7 days, there was a slight increase in fibrosis in the media (1.0±0.2) and adventitia (0.5±0.2). The medial layer displayed evidence of a complete healing process through fibrosis with a decrease in medial hyalinization (0.5±0.4) and complete endothelialization (4.0±0.0). Distal nerve atrophy remained a key histological feature indicative of treatment effectiveness (1.2±0.3).

Neuromatous regeneration was apparent (0.1±0.1) in conjunction with a significant increase in nerve perineurial and endoneurial fibrosis (1.5±0.3) and with a decrease in nerve inflammation (0.1±0.2) at treated levels. Neuromatous regeneration was characterized by disorganized sprouting of neuroid fibers within the thickened perineurium. At 30 days, there was weak to absent TH and S100 staining in most nerves except for fibers and regenerative sprouts in the thickened perineurium of nerves directly within the RF lesion. However, all nerves, except atrophic nerves, showed strong GAP43 staining particularly in perineurial sprouts and neuromatous tangles within the fibrous perineurium.

Healing and regeneration progressed further by 90 days. The average depth of RF changes was slightly reduced (2.6±1.0mm) compared to previous time points consistent with more advanced healing, whereas percentage of circumferential extension remained expectedly largely unchanged (46±23%). The medial vascular layer showed full recovery with no hyalinization (0.0±0.0) and complete endothelialization (4.0±0.0), with a comparable thickness at the ablation site of 338±164 µm and 364±72 µm at the unexposed site. There was no arterial wall inflammation observed (0.0±0.0), a slightly higher mean fibrosis in the media (1.3±0.7) and persistent fibrosis in adventitia (0.5±0.2) compared to previous time points.

High levels of perineurial nerve fibrosis (1.4±0.4) remained and was associated with higher levels of neuromatous regeneration (0.8±0.7). Distal nerve atrophy was attenuated compared to Day 30 (0.3±0.2) and nerve inflammation was completely resolved (0.0±0.0). At 90 days, there was some degree of recovery of TH expression in the periaerial renal nerves compared to earlier time points, although staining remained uneven and somewhat weaker than in the untreated controls. There were also prominent neuromatous tangles with disorganized architecture that showed strong S100 and GAP43 staining (Figure 5 and 6).

Two Radiofrequency Treatment Cycles (Day 7 only)

There were no appreciable or biologically meaningful differences in the histological features following the delivery of two radiofrequency treatment cycles at each location compared to the arteries that received only one treatment cycle. The same relative depth of RF changes was observed with two treatment cycles (3.9±0.5mm) compared to one treatment cycle (3.6±0.8mm). The same relative percentage of circumferential arterial involvement was observed in both treatment groups (one cycle=40±13% vs two cycles=47±14%). In neither treatment group was inflammation or medial or adventitial fibrosis observed (0.0±0.0). Regardless of the therapy, no differences in medial hyalinization (one cycle=1.0±0.4 vs two cycles=1.1±0.3) or in endothelialization (one cycle=3.9±0.2 vs two cycles=4.0±0.1) were observed. RF treatment depth reached 8.5 mm in the two treatment cycle group. Nerve changes were very similar; nerve inflammation (one cycle=0.5±0.3 vs two cycles=0.4±0.3), nerve atrophy (one cycle=1.1±0.7 vs two cycles=1.0±0.4), neuromatous regeneration (one cycle=0.0±0.0 vs two cycles=0.1±0.2) nerve perineurial and endoneurial fibrosis (one cycle=0.5±0.3 vs two cycles=0.6±0.3) were virtually the same and nerve necrosis was slightly higher in the two-cycle group (one cycle=0.7±0.3 vs two cycles=1.2±0.5; Figure 5 and 6).

Histological correlation with Norepinephrine levels

The level of NE was correlated with the percentage of circumference displaying evidence of thermal ablation. As expected, there was a significant negative correlation with the circumferential extension (Figure 7a); the higher the percent of wall circumferential extension, the lower level of NE level (r=−0.43, p<0.05). Distal nerve atrophy is one of the main histological markers of an effective delivery of thermal energy to more proximal nerves. When correlated with the levels of NE concentration, there was a statistically significant negative correlation (r=−0.52, p<0.01; Figure 7b). Importantly, for renal arteries that displayed the highest amount of distal nerve atrophy, the kidneys demonstrated the lowest levels of NE; supporting the consideration of distal nerve atrophy as a key histological marker of effective renal nerve ablation. Furthermore, at 90 days when fibrosis and the neuromatous regeneration were more prominent, this same negative correlation persisted. A significant negative correlation also existed between perineurial/endoneurial fibrosis (r=−0.88, p<0.01; Figure 7c) or neuromatous regeneration (r=0.83, p<0.01) and the levels of NE (Figure 7d). These negative correlations also point to features relating to the efficiency of the thermal ablation of the nerves histologically and a physiologic disruption of the SNS.

Discussion

Based on the gross and microscopic evaluation of the renal arteries, renal parenchyma, and clinical evaluation of the animals that received radiofrequency energy for renal nerve ablation, we conclude that the Iberis™ Renal Denervation System is considered to be safe as tested in a porcine model. The results presented in this study demonstrate that the delivery of RF energy results in histopathological changes in the vessel wall involving 40% to 100% of the circumference of the artery with an average penetration depth of 3.6±0.8 mm, 3.3±1.1 mm, and 2.6±1 mm at 7 days, 30 days, and 90 days, respectively. Angiography showed no luminal flow disturbances nor stenosis at any time points. Histology showed no substantial presence of neointimal hyperplasia despite the extensive to complete circumferential involvement. The main histopathological features observed at 7 days (necrosis, media hyalinization, distal nerve atrophy) were followed by a progressive healing response at 30 and 90 days (arterial and perivascular as well as nerve fibrosis and
neuromatous regeneration). These features are consistent with the current literature describing the histopathological observations following RF ablation with other devices.\textsuperscript{14,17,18}

Effective RF nerve ablation has been previously shown to decrease NE levels in large animal models.\textsuperscript{19,20} In this study, the norepinephrine levels measured in the single ablation animals demonstrated a significant NE decrease at all time points compared to naïve kidneys. The dramatic drop at 7 and 30 days, interestingly, is attenuated at 90 days, albeit still statistically decreased compared to controls. The ability of nerves to sprout and attempt to regenerate in response to injury has been previously described.\textsuperscript{21–23} This study strongly suggests that renal sympathetic nerves attempt to regenerate but without apparent functional reconnection based on persistent NE level suppression.\textsuperscript{14,24–26} The high degree of distal nerve atrophy observed at 7 and 30 days in this study reported at chronic time points in other large animal experiments where RF ablation of renal nerves was performed.\textsuperscript{14,24} It is necessary to further establish if the apparent morphological “recovery” of distal nerves could be responsible for the late increase in NE levels and whether this partial recovery may or may not produce a physiologic increase in blood pressure. The available clinical data though suggest sustained blood pressure lowering effects up to 36 months following the procedure.\textsuperscript{21,26}

As described earlier, the decrease in NE levels show a statistically significant negative correlation with the percentage of circumference of vascular wall involvement, distal nerve atrophy, perineurial/endoneurial fibrosis and nerve neuromatous regeneration, the last three being the main histopathological markers of an effective RF nerve ablation. This negative correlation offers some surrogate histological endpoints as assurance that an effective physiologic disconnection is established. Concordant with existing studies,\textsuperscript{16} the immunohistochemistry results in our study showed that ablated nerves display decreased staining for tyrosine hydroxylase (TH) and S100 at 7 and 30 days, with some partial recovery of TH staining at 90 days. The regenerative activity was evidenced by the presence of GAP43 at all time points. In our study, the delivery of one or two ablation cycles did not produce any angiographic or histopathologic safety concerns. In addition, there were no histopathological differences between one or two ablation cycles in the artery or in the surrounding tissues. When comparing one to two cycles, there were no or only marginal increases in depth, in percent circumference involvement, or in degree of nerve ablation or distal atrophy. Extrapolation of this translational information suggests that if operators perform overlapping or repetitive ablation in humans, there is no significant associated safety risk but also no added value with respect to greater penetration depth based on the data in the swine model. This is in concordance with other preclinical data.\textsuperscript{23}

**Limitation of the Study**

It is important to note that the scope of this study was to evaluate the safety of the Iberis™ RDN system. For this reason, efficacy (NE levels) was not evaluated in all groups (exclusion of NE evaluation for the two ablation cycle arm at 7 days). The evaluation of NE levels following delivery of 8 watts of energy for 120 seconds (single ablation) provides insight into the efficacy of RF ablation in normotensive swine. This marker has been utilized by numerous groups to assess the efficacy of renal denervation using various energy sources including RF,\textsuperscript{4,15,22} Ultrasound,\textsuperscript{25} and Cryoablation.\textsuperscript{23} In this study, immunohistochemistry was performed to characterize the nerve response following RDN at time points up to 90 days. Longer time points may be required, since the potential for regenerative activity to restore function remains unclear. While the restoration of nerve physiology seems histologically unlikely due to the disrupted architecture of the neuromatous tangles at the RF lesion sites (neuromatous regeneration), this needs to be evaluated at longer term chronic follow up and the translation of these findings to humans remains to be determined.\textsuperscript{24}

**Conclusion**

The Iberis™ renal denervation system was shown to be safe following one or two cycles of RF ablation at multiple sites in the main renal artery of swine. Renal cortical norepinephrine decrease was demonstrated at 7, 30, and 90 days and correlated negatively with histological markers of nerve damage. These parameters indicate that RF treatment is an effective modality of nerve ablation in this model, consistent with the intended human indication for use.

**Disclosures**

Author AS is a full time employee of Terumo Corporation. All other authors have no disclosure or conflict of interest to present in regards to the data presented in this manuscript.

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**Abbreviations**

RDN- Renal Denervation
RF- Radiofrequency
NE- Norepinephrine
IHC- Immunohistochemistry
SNS- Sympathetic nervous system
AAALAC- Association for Assessment and Accreditation of Laboratory Animal Care
IV- intravenous
IM- intramuscular
KCL- Potassium Chloride
H&E- Hematoxylin and Eosin
ET- Elastin Trichrome
TH- Tyrosine Hydroxylase
GAP43- Growth Associated Protein 43
S100- Schwann cell 100
TIMI- Thrombolysis in Myocardial Infarction grade flow

**References**
