Journal of Controlled Release xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel



# *In vivo* evaluation of the delivery and efficacy of a sirolimus-laden polymer gel for inhibition of hyperplasia in a porcine model of arteriovenous hemodialysis graft stenosis

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ARTICLE INFO

*Article history:*
Received 1 February 2012
Accepted 11 March 2012
Available online xxxx

*Keywords:*
Polymer gel
Sustained drug delivery
Sirolimus
MRI
Hemodialysis vascular access
Stenosis

ABSTRACT

Synthetic arteriovenous (AV) hemodialysis grafts are plagued by hyperplasia resulting in occlusion and graft failure yet there are no clinically available preventative treatments. Here the delivery and degradation of a sirolimus-laden polymer gel were monitored *in vivo* by magnetic resonance imaging (MRI) and its efficacy for inhibiting hyperplasia was evaluated in a porcine model of AV graft stenosis.

Synthetic grafts were placed between the carotid artery and ipsilateral jugular vein of swine. A biodegradable polymer gel loaded with sirolimus (2.5 mg/mL) was immediately applied perivascularly to the venous anastomosis, and reapplied by ultrasound-guided injections at one, two and three weeks. Control grafts received neither sirolimus nor polymer. The lumen cross-sectional area at the graft–vein anastomosis was assessed *in vivo* by non-invasive MRI. The explanted tissues also underwent histological analysis.

A specifically developed MRI pulse sequence provided a high contrast-to-noise ratio (CNR) between the polymer and surrounding tissue that allowed confirmation of gel location after injection. Polymer signal decreased up to 80% at three to four weeks after injection, slightly faster than its degradation kinetics *in vitro*. The MR image of the polymer was confirmed by visual assessment at necropsy. On histological assessment, the mean hyperplasia surface area of the treated graft was 52% lower than that of the control grafts (0.43 $mm^2$ vs. 0.89 $mm^2$; $p<0.003$), while the minimum cross-sectional lumen area, as measured on MRI, was doubled (5.3 $mm^2$ vs 2.5 $mm^2$; $p<0.05$).

In conclusion, customized MRI allowed non-invasive monitoring of the location and degradation of drug delivery polymer gels *in vivo*. Perivascular application of sirolimus-laden polymer yielded a significant decrease in hyperplasia development and an increase in lumen area at the venous anastomosis of AV grafts.



## 1. Introduction

A reliable vascular access providing high blood flow rates is critical for chronic hemodialysis. Access for hemodialysis is preferably achieved through the creation of a native fistula, where a vein is connected to an artery to receive arterial flow, or through the placement of a synthetic graft between the artery and vein. In contrast to the native arteriovenous (AV) fistula, the synthetic AV graft quickly and more reliably produces an access with sufficiently high blood flow rates necessary for hemodialysis; however synthetic grafts suffer from a high rate of stenosis due to aggressive hyperplasia development typically at the anastomosis between the vein and graft [1]. The native AV fistulas have a lower incidence of stenosis and clotting than synthetic AV grafts. However, fistulas sometimes cannot be created due to inadequate blood vessel size or condition, and up to 60% of fistulas fail to develop sufficient blood flow to achieve dialysis [2]. If the primary patency of the synthetic AV graft could be significantly prolonged, these conduits would prove an attractive alternative to the native fistula.

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doi:10.1016/j.jconrel.2012.03.011

There are currently no clinically available preventative treatments for AV graft stenosis. Of note, in a recent clinical study, the placement of polytetrafluoroethylene-encapsulated nitinol stents after balloon angioplasty of stenotic lesions in synthetic AV grafts was shown to be associated with significantly less restenosis than balloon angioplasty alone [3]. However, the rates of subsequent thrombotic occlusion were similar between the two groups and even with stent placement, approximately one third of treated grafts were lost to thrombosis. Additionally, the primary patency of the vascular access after stent placement was less than 40% at six months, indicating that preventive therapies are still urgently needed to prolong AV graft patency.

Sirolimus (rapamycin) is a macrolide antibiotic that is administered orally for preventing rejection of organ transplants and is employed in drug-eluting coronary stents for targeted intraluminal delivery after angioplasty to inhibit restenosis. Drug-eluting stents have the disadvantage in that the drug is usually exhausted within approximately three months of application with no means of replenishment. Angioplasty to treat stenotic lesions is a solitary insult to the vascular wall; therefore, the limited duration of release of anti-proliferative drugs from a drug-eluting stent may be a reasonable strategy to prevent restenosis. In contrast, the hemodialysis access suffers long-term frequent insults as it is punctured repeatedly for thrice-weekly dialysis sessions. The repeat needle punctures instigate platelet activation and thrombosis with subsequent release of growth and migratory factors such as platelet-derived growth factor and fibroblast-growth factor. In addition, the continual presence of the foreign material of the graft itself promotes inflammation and vascular cell proliferation. Lastly, the end-stage renal disease population has unique risk factors for stenosis such as uremia and hypercoaguability [2,4]. Consequently, the unique characteristics of the AV graft and its abnormal physiological environment may require *repeat* sustained drug delivery to inhibit stenosis during the lifetime of the access.

Superficial hemodialysis grafts are readily accessible percutaneously. Thus, the placement of a perivascular depot could be a straightforward technique to provide sustained delivery of anti-proliferative drugs for inhibition of vascular hyperplasia. We previously reported that serial injections of a sustained delivery, biodegradable, biocompatible, polymer gel (ReGel™) loaded with sirolimus provided high, localized, concentrations of drug at target vascular tissues in a porcine model of AV graft stenosis [5]. Here, we extend that work and report the *efficacy outcome*. We show that serial injections of the drug-laden polymer inhibited hyperplasia development in the porcine model of AV graft stenosis. We also report the use of MRI for the *in vivo* tracking of the polymer gel location and gel degradation as well as assessment of vascular lumen and development of hyperplasia.

## 2. Materials and methods

### 2.1. Porcine model of AV graft stenosis

Yorkshire cross-domestic pigs were used for graft implantation as previously described by our laboratory [6] and as described in detail in the Online Data Supplement. Briefly, under sterile conditions, a 7-cm length of expanded polytetrafluoroethylene (ePTFE) graft (Bard Peripheral Vascular Inc., Tempe, AZ) was placed between the common carotid artery and the ipsilateral external jugular vein to create a graft-end-to-vessel-side anastomoses so that arterial blood flow was shunted into the vein (Fig. 1). Blood samples were drawn for basic blood chemistries (Antech Diagnostics, Salt Lake City, UT) and plasma sirolimus levels (ARUP Laboratories, Salt Lake City, UT).

Animals were randomly assigned to receive local sirolimus treatment (treated group) or no treatment to the graft (control group). Sirolimus powder (LC Laboratories, Woburn, MA) was mixed *in vitro* with a liquid polymer gel (see below), to yield a final concentration of 2.5 mg/mL. This concentration was chosen based on a previous pharmacokinetic study demonstrating that sustained tissue drug concentrations, above the $IC_{50}$ for inhibiting smooth muscle cell proliferation, were achieved in the anastomotic tissue [5]. Additionally in the pharmacokinetic studies, this dose was well tolerated. The polymer–drug suspension was maintained at 4 °C until its application to the venous anastomosis of the AV graft in the pig. No polymer or sirolimus was applied to animals in the control group.

All animal work was performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Utah and Veterans Affairs Salt Lake City Healthcare System and conformed to the guidelines established by the *Guidelines for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).

### 2.2. Polymer gel

The polymer used for these studies is a thermosensitive biodegradable ABA triblock copolymer, with an average molecular weight ($M_{av}$) of approximately 4200. The A block is poly(D,L-lactide-co-glycolide) and the B block is polyethylene glycol (PEG) (ReGel™, Protherics Salt Lake City, Inc., a BTG Company). The polymer is prepared by a ring-opening of D,L-lactide, glycolide and PEG initiated by addition of stannous octoate and partially characterized by $^1$HNMR and gel permeation chromatography [7]. This polymer exists as an aqueous liquid (sol) state when below its gelation temperature (15 °C) and in this form can be mixed with drug. It transitions spontaneously into a semi-solid state above 15 °C, serving as a localized, sustained-release drug depot at the site of application. The polymer degrades over a period of weeks, into lactic acid, glycolic acid and PEG. We previously studied the *in vivo* release kinetics of sirolimus from this gel system and reported that, using the application protocol described in Table 1, levels of sirolimus

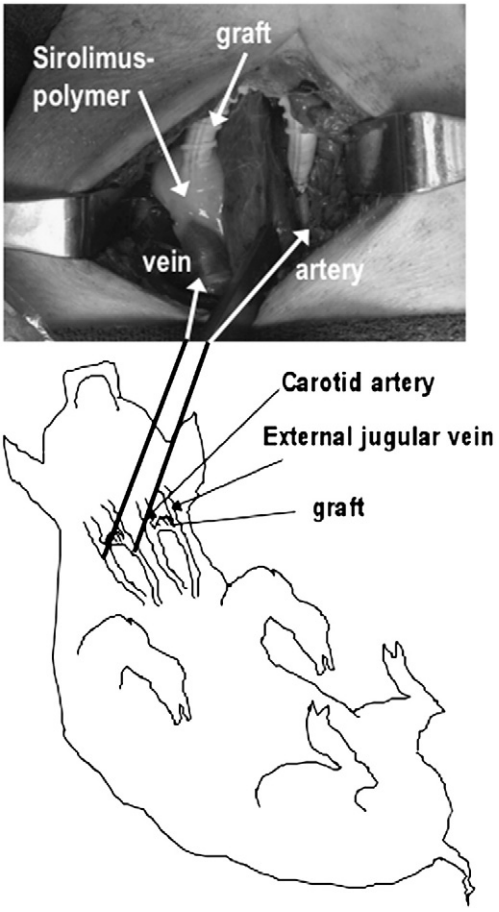


**Fig. 1.** Porcine model.



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could be achieved in the venous anastomotic tissue that were significantly greater than concentrations needed to inhibit smooth muscle cell proliferation *in vitro* [5].

### 2.3. Ultrasound (US)-guided injection of sirolimus-laden polymer

Besides the direct intra-operative application to the venous anastomosis, the drug-treated animals also received US-guided injections of 2 mL of liquid sirolimus-laden polymer (2.5 mg/mL) at the anastomotic perivascular region of the vein graft (see Fig. 2) at one, two and three weeks after graft placement (see Table 1). A sequence of US images taken before and after injection of the polymer to the perivascular area of the graft–vein anastomosis in one animal is shown in Fig. 2. An echogenic signal occurred upon injection but shadows were cast by the gel that prohibited visualization of the blood vessel and other structures beneath the gel. This hindered assessment of the spatial relationship between the graft and blood vessels and the injected polymer. Lastly, in contrast to MR images shown below, when the injection sites were examined by US one week later, very little echogenicity was typically observed (not shown), suggesting that the sensitivity of US in detecting polymer gels at the perivascular region is limited. After injection, attempts were made to locate gel by gross visual inspection of the surgical cavity after reopening of the wound. However, as the gel is opaque and the wound area contained sera/exudate and clot up to two weeks postoperatively that obscured the view, these attempts were not successful. Polymer was also injected into the muscle above the graft in one animal as described in the Results section. Animals were subjected to weekly AV graft patency monitoring using color Doppler US.

### 2.4. In vivo MRI for visualization of polymer gel and AV graft

*In vitro* MRI experiments were performed to determine the appropriate parameters that were used to differentiate polymer gel from sera in MR images (described in detail in the Online Supplement). For *in vivo* MRI, sedated animals were transported to the MR scanner (3T Trio, Siemens Medical Solutions, Erlangen, Germany) within 1 h after injection of the polymer. The animals were placed in a supine position within the bore of the magnet. Images were obtained using an overlap-decoupled 16-channel phased-array radio frequency (RF) coil mounted on a fiberglass support molded to fit the porcine neck, placed directly over the surgical site, or with eight total coil elements forming two sets of bilateral pair array coils. A localization scan was performed using axial 2D time-of-flight (TOF), with echo time (TE) of 5.9 ms and repetition time (TR) of 25 ms. A T2-weighted (T2w) 3D TSE sequence with restore pulse, and imaging parameters that were specifically optimized for the gel visualization (TE/TR = 142/550 ms, echo train length (ETL) = 17, voxel dimensions $0.7\times0.7\times0.7$ mm$^3$, FoV $186\times223$, acquisition time 12 min), were applied to image the polymer gel. A T1-weighted 2D TSE black-blood sequence (TE/TR = 8.6/800 ms, inversion time (TI) = 500 ms, echo train length (ETL) = 9, voxel dimensions $0.6\times0.6\times2.0$ mm$^3$, Field of View (FOV) $180\times192$, acquisition time 10 min) and a 3D TSE black-blood sequence (TE/TR = 11/665 ms; ETL = 17; voxel dimensions, $0.5\times0.5\times0.5$ mm$^3$; FOV, $192\times156$, acquisition time of 12 min) were applied to visualize the graft and blood vessel lumens [8].

### 2.5. Calculation of signal-to-noise ratio (SNR) and contrast-to-noise ratios (CNR) of polymer signal

An MR image slice containing polymer signal was chosen from all image slices available in the T2w polymer-optimized imaging series available for that injection. Using MatLab software (Mathworks, Inc., Natick, MA), a region of interest was encircled within the polymer signal and average pixel signal intensity was evaluated. SNR for each *in vivo* polymer image was determined by dividing the average polymer pixel intensity by the noise (see Online Supplement) obtained in the same image slice. SNR for background tissue near the polymer was determined similarly. CNR for each polymer image was determined by subtracting the SNR of the background from the SNR of the polymer. The SNR and CNR values for each DICOM image were averaged to yield an average and standard deviation SNR and CNR.

### 2.6. MR image analysis of venous anastomosis and juxta-anastomotic tissues

OsiriX medical image processing software (Osirix v. 3.2.1), an open-source software (http://www.osirix-viewer.com/Downloads.html), was used for MR image analysis by three individual investigators (CT, HL, IZ) who were blinded to animal treatment at the time of analysis. The narrowest cross-sectional lumen area at the anastomosis of the vein and graft was measured by each investigator (see Fig. I in Online Supplement) and the video file (See Video 1 in Online Supplement). The measured lumen cross-sectional area was then normalized by dividing by the total graft cross-sectional area (including the graft wall and lumen) and multiplying by 100 and expressed as a percent of graft area. All MRI data, with the exception of the 2D analysis of one control animal, was deemed acceptable and was included in the analysis.

### 2.7. Number of animals

At the beginning of this series of experiments, bilateral AV grafts were placed in each animal. However, upon approval by the animal care and use committee, the experimental protocol was subsequently converted to unilateral grafts in order to decrease the surgical and post-operative stress on the animals. In the final analysis, there were eleven animals in the sirolimus-treated group (see Table 2) with five animals receiving bilateral grafts and six receiving unilateral grafts. There were twelve animals in the control group with nine animals receiving bilateral grafts and three receiving unilateral grafts. When a single graft was placed in an animal, the side receiving the graft was chosen at random. A total of 51 grafts were placed in 32 animals. However, animals in which the grafts were occluded within three weeks of surgery (five animals) or animals that were euthanized for any cause prior to the pre-determined six-week endpoint (four animals) were excluded from analysis. The decision to exclude such animals was made prior to examination of the raw data. The rationale for excluding the

**Table 1**
Study protocol for treated and control groups.

| Time point | Day 0 | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
|---|---|---|---|---|---|---|---|
| *Treated* | Blood collection<br>Graft surgery<br>Drug-polymer application | Patency check<br>Drug–polymer injection | Patency check<br>Drug–polymer injection | Patency check<br>Drug–polymer injection | Patency check | Patency check | MRI<br>Blood collection<br>Euthanasia |
| *Control* | Blood collection<br>Graft surgery | Patency check | Patency check | Patency check | Patency check | Patency check | MRI<br>Blood collection<br>Euthanasia |

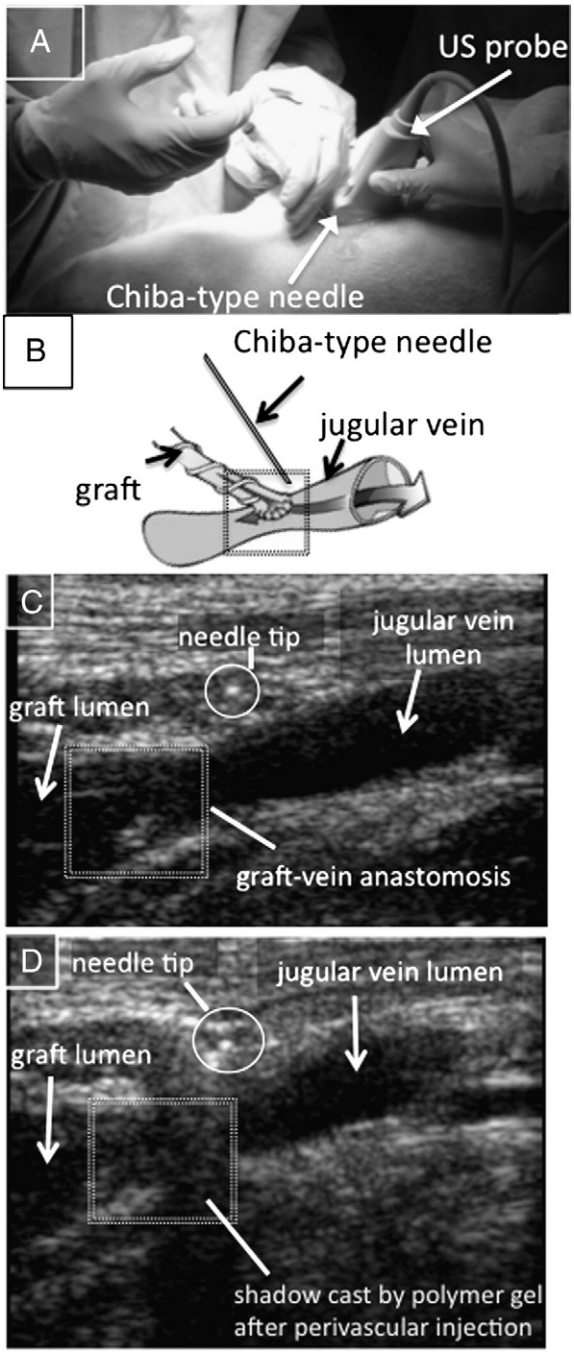


**Fig. 2.** Ultrasound (US)-guided injection of sirolimus-laden polymer gel. (A) A Chiba-type needle was inserted at the graft–vein anastomosis perivascular region under sterile conditions while imaging with an US probe. (B) A drawing showing the position of the Chiba-type needle in relation to the graft–vein anastomosis after US-guided placement. Arrow in vein depicts blood flow. (C) A US image of the needle and graft–vein anastomosis just prior to polymer gel injection. (D) A US image of the graft–vein anastomosis obtained during polymer injection.

early occlusion cases was that the occlusion would likely be attributable to apparent technical problems as no significant hyperplasia could be demonstrated at these early time points. Only animals that were maintained to the six-week endpoint were included in the final analysis.

### 2.8. Explantation of venous anastomosis and juxta-anastomotic tissues

With the animal under general anesthesia, the AV graft and attached native vessels were ligated. The lumens of the ligated vessels were rinsed with saline, and perfused with 10% zinc-formalin to fix the anatomical structures and maintain the lumen circumference. After euthanasia, the grafts and ligated vessels were explanted en bloc and fixed in formalin overnight, paraffin embedded and subjected to morphometric analysis as illustrated in Fig. 3 and described in the Online Supplement [6].

### 2.9. Statistical analysis

The software package SAS (v9.2) was used for statistical analysis. Mean ± SD values were calculated for continuous variables. A one-sided Wilcoxon two-sample test was used to test differences i) between the H/G ratios (ratios between the cross-sectional area of the neointimal hyperplasia and the cross-sectional area of the AV graft) calculated from graft histology sections (heel, middle and toe sections) obtained from treated and control animals, and ii) between the lumen cross-sectional areas at the graft–vein anastomosis of treated and control animals as determined in MR images. To test the difference in occlusion rates of grafts between treated and control, a chi-square contingency table analysis was done. ANOVA was used to test differences in blood chemistries between pre-operative values and values at six post-operative weeks for control or treated animals.

## 3. Results

### 3.1. Study protocol and summary of morbidity and mortality

A summary of the study protocol is provided in Table 1. As indicated in Table 2, a total of 23 animals with 32 grafts were used in the final analyses of hyperplasia. Also presented in Table 2 is a summary of the morbidity, mortality as well as early and late graft occlusions. There was no significant difference in early occlusion rates between the treated and control groups (23.1% vs. 26.1% respectively; $p = 0.81$). There was a trend toward fewer late (after the 3rd week) occlusion events in the treated grafts compared to controls but this did not reach significance (3.6% vs. 21.7%; $p = 0.178$).

Five of the 18 treated animals experienced some localized swelling and exudate near the graft. In two of these five animals, the swelling was observed at five and six weeks after surgery respectively but was well tolerated and not associated with anorexia or weight loss. These two animals were maintained to the six-week close-out time point and included in the final analyses for hyperplasia. Three of the five animals experienced marked swelling within two weeks of graft placement that affected the animals' well-being. These three animals were euthanized before the planned six-week time point and were excluded from the final analyses for hyperplasia. Gram stain of the exudates showed very few leukocytes and no bacteria (data not shown). No bacteria were cultured from the exudate tested. In contrast, no remarkable local swellings were observed in any control animals. In a separate series of study, the polymer gel alone without sirolimus was applied to the venous anastomosis in five animals, with no apparent untoward local or systemic effects observed up to four weeks (data not shown).

### 3.2. MR imaging of polymer gel depot in vivo

MR with pulse sequences optimized to capture the polymer gel was used to monitor the localization of the US-guided injection of the gel. The mean contrast-to-noise ratio (CNR) of the polymer from MR images of 17 injections was 82.6 ± 44.8, indicating that our optimized pulse sequence yielded excellent visualization of the polymer gel. Fig. 4 shows the typical results of an MR scan obtained directly after injection. Fig. 4B shows an MR image obtained immediately after gel injection using the polymer-optimized scan parameters and shown in the transverse (axial) plane. The image shows that, in this animal, the gel was injected perivascular to the external jugular



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**Table 2**
Summary of morbidity, occlusion and early mortality in treated and control groups.

| | Initial no. of pigs (grafts)[a] | Grafts with early occlusion[b] | Pigs with early occlusion in all grafts[c] | Pigs (grafts) with wound swelling[d] | Pigs (grafts) with complications[e] | Pigs (grafts) in final analysis | Pigs (grafts) with late occlusion[f] |
|---|---|---|---|---|---|---|---|
| Treated | 18 (28) | (6)[g] | 3 | 3 (5) | 1 (2) | 11 (15) | 1 (1) |
| Control | 14 (23) | (6)[g] | 2 | 0 | 0 | 12 (17) | 4 (5) |

[a] There are more grafts than pigs because some animals received bilateral grafts.
[b] These grafts were not included in the final hyperplasia analysis because occlusion occurred within three weeks of surgery, when significant hyperplasia was absent. Nine control animals had bilateral grafts, and 4 of those animals had 1 graft experiencing early occlusion (4 of the 6); five treated animals had bilateral grafts, and 1 of those animals had 1 graft experiencing early occlusion (1 of the 6). Other occlusions occurred in unilateral grafts.
[c] These animals had occlusion within three weeks of surgery in either their only graft or in both grafts, thus these animals were not included in the final analysis. The grafts from these animals are included in the "graft with early occlusion" column to the left.
[d] Three animals experienced marked exudate and swelling after polymer/drug application and were euthanized before the six-week time point.
[e] One treated animal was lost before the six-week time point due to accidental injection of polymer into the vein at 3 weeks after surgery.
[f] Late occlusion was defined as occlusion that occurred after three weeks of graft placement. These animals were included in the final analysis.
[g] These grafts were not included in the final hyperplasia analysis.

vein. The same animal was imaged again one week after injection whereupon the polymer signal was still visible but appeared to have diffused slightly toward the skin surface and underneath the vein (Fig. 4C). Further, the signal intensity decreased, suggesting that the gel had undergone compositional changes, such as a decrease in mass due to diffusion of low molecular weight components of the polymer, during this time interval. We measured the changes in CNR of polymer depots over time in seven animals. In five of these animals, the CNR decreased by between 55% to 80% three or four weeks after the initial injection (see Fig. II in the Online Supplement). By comparison, when the polymer gel was incubated in 2% bovine serum albumin in saline at 37 °C with sink conditions, almost complete polymer breakdown was observed by six weeks.

The majority of swelling and exudate that occurs with the initial surgery typically subsided within five days after graft placement but occasionally polymer was injected before the swelling had completely subsided. In such an instance the gel-optimized pulse sequence showed the presence of a bright signal where no polymer had been injected. When the area was examined on images collected using a black-blood pulse sequence, the bright signal was markedly diminished but the polymer signal still retained a higher CNR (Fig. III in the Online Supplement). *In vitro* studies of polymer and serum showed similar T1 and T2 relaxation times (Table I Online Supplement). This suggested that the bright signal observed in absence of polymer injection was likely due to the presence of serum exudate still present from the surgical wound. In further support, when such an animal was imaged at later time points, the bright signal, purportedly from serum/exudate, had largely resolved but signal from polymer, was still apparent (Fig. IV Online Supplement). This data indicates that polymer gel can be differentiated from serum exudate by MRI.

### 3.3. Validation of MR image of injected polymer gel by gross visual examination

To further confirm the veracity of MR imaging of the polymer, we compared MR image with direct visual inspection. To that end, a highly lipophilic and hydrophobic dye (Sudan Black) was mixed with the liquid polymer and injected at the perivascular anastomotic region nine days after graft placement without US guidance. Fig. 5 shows MR images obtained before (Fig. 5A) and after (Fig. 5B). Fourteen days later, the injection site was exposed surgically for gross inspection. The location and shape of the dye visualized grossly (Fig. 5C) were very similar to those indicated by the MRI. These data further validated the accuracy of MRI with optimized pulse sequences for monitoring the polymer gel *in vivo*.

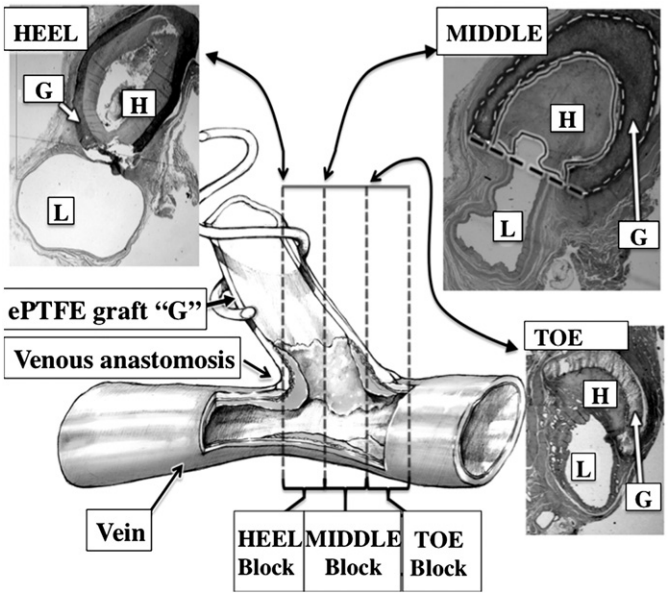


**Fig. 3.** Tissue sampling at the AV graft for morphometric analysis. In the "middle" histology section, the ePTFE graft surface area "G" is outlined by a white dashed line; the hyperplasia surface area "H" is outlined by a thin solid black line; the mouth of the graft is delineated by a dashed black line to demonstrate the H/G ratio technique. "L" is the vein lumen. The spiral reinforcement is shown surrounding the graft in the cartoon.

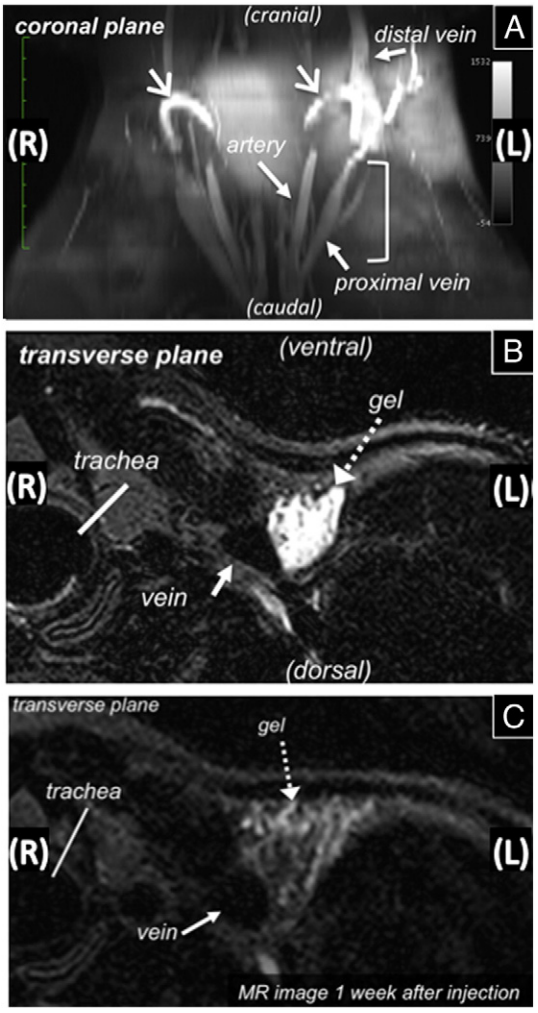


**Fig. 4.** MRI of the polymer location and degradation. (A) An axial 2D TOF image of the porcine neck. Grafts are indicated by the open-head arrows. Polymer was injected at the region indicated by the bracket. (B) An MRI of the neck of the same animal shown in panel A, obtained immediately after injection using a T2-w polymer-optimized sequence showing the polymer in the transverse (axial) plane near the jugular vein (labeled as "vein"). (C) MRI obtained from the same animal one week later.

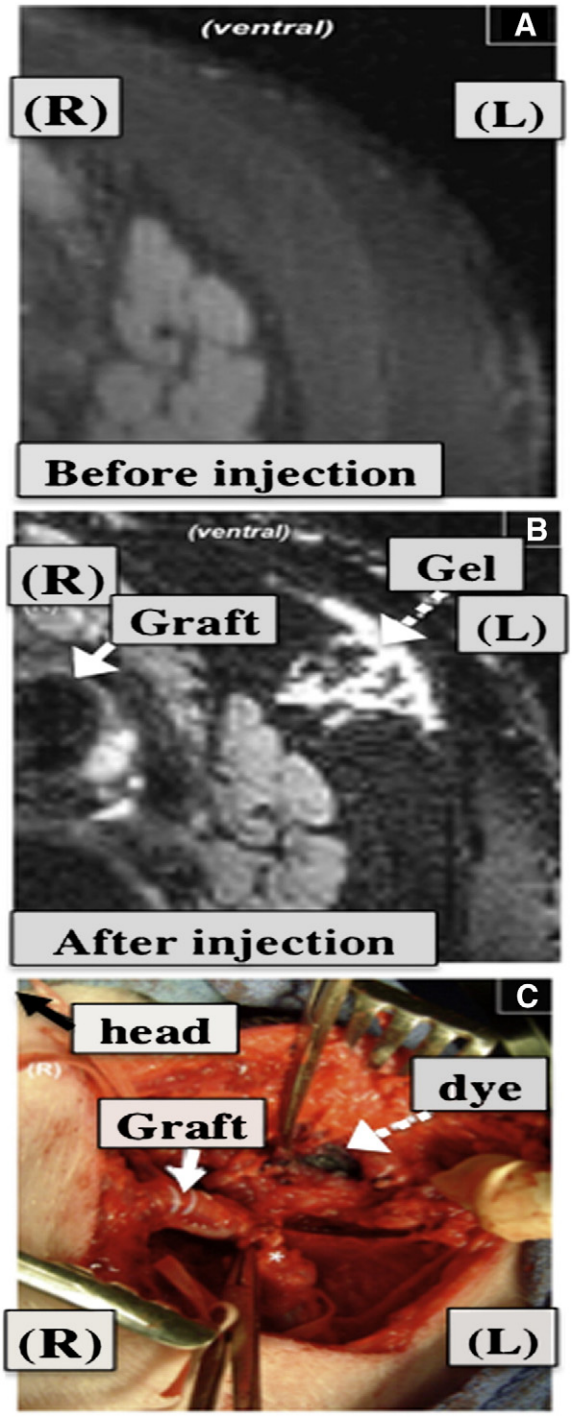


**Fig. 5.** Validation of the polymer MR image by gross visual inspection. (A) A transverse plane of an MR image obtained immediately before and (B) after injection of the polymer (indicated by the dotted arrow) into a muscle near the graft. (C) Surgical exposure of the area 14 days after injection revealed the dye (dotted arrow) to be in the same location.

### *3.4. Perivascular sirolimus treatment inhibits AV graft hyperplasia*

Hyperplasia at the anastomosis of the vein and graft was quantified on histological slides of cross-sections at the "toe", the "middle" and the "heel" regions of explanted tissues (refer to Fig. 3 in Materials and methods). The results are shown in Fig. 6. Perivascular sustained delivery of sirolimus was associated with significantly decreased hyperplasia in the middle sections of the graft, compared to the control (0.43 $mm^2$ hyperplasia per $mm^2$ of graft area vs. 0.89 $mm^2$ hyperplasia per $mm^2$ of graft area; $p<0.003$).

### *3.5. Perivascular sirolimus treatment increases AV graft lumen area*

All 11 sirolimus-treated animals and eight of 12 control animals underwent MRI. Lumen cross-sectional areas in both 2D- and 3D-black-blood MR images were measured. (An example is shown in Fig. I of the Online Supplement). The results are shown in Fig. 7 and, as a normalization factor, are expressed as percentages of the graft cross-sectional areas obtained in the same location. Perivascular sustained delivery of rapamcyin was associated with a lumen cross-sectional area that was significantly increased, compared to control animals. When expressed without normalization, the average lumen area of the treated grafts was 5.3 $mm^2$ vs. an average lumen area of 2.5 $mm^2$ for the control grafts $p<0.05$).

### *3.6. Other outcomes*

At the end of the six-week study period, systemic plasma sirolimus levels were below the assay detection limit of 2 ng/mL in six of the ten animals that received perivascular sustained delivery of sirolimus

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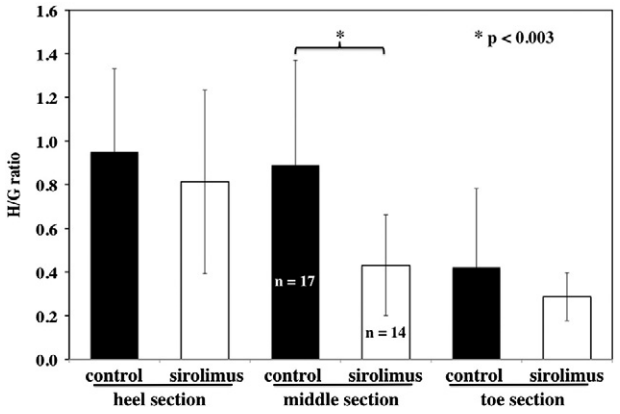


**Fig. 6.** Perivascular treatment with sirolimus is associated with decreased hyperplasia formation at the venous anastomosis. Histological cross-sections from "toe", "middle" and "heel" regions at the venous anastomosis of sirolimus-treated grafts and control grafts underwent morphometric analysis. The cross-sectional surface area of the hyperplasia (H) was normalized by the cross-sectional surface area of the graft (G) and expressed as the H/G ratio. Each bar represents mean ± SD.

and underwent blood draw for the drug assay. In the other four treated animals, the plasma sirolimus levels were slightly above the detection limit and ranged from 2.75 ng/mL to 3.6 ng/mL (Table 3). Sirolimus was undetectable in the plasma of all seven control animals that were tested. No statistically significant differences in blood chemistries were observed between the treated and control animals at six weeks after graft placement (Table 3).

## 4. Discussion

Our previous study employing the same approach in the same porcine AV graft model showed that repeat perivascular application of sirolimus-loaded polymer gel resulted in clinically relevant levels of drug in the venous anastomotic tissues [5]. The data presented in the current study indicated that this approach was indeed effective in the inhibition of stenosis in AV grafts. At the end of the six-week period after graft placement, the average cross-sectional hyperplasia surface area in the middle section of the venous anastomosis was decreased by more than 50%, compared to the control grafts (Fig. 6).

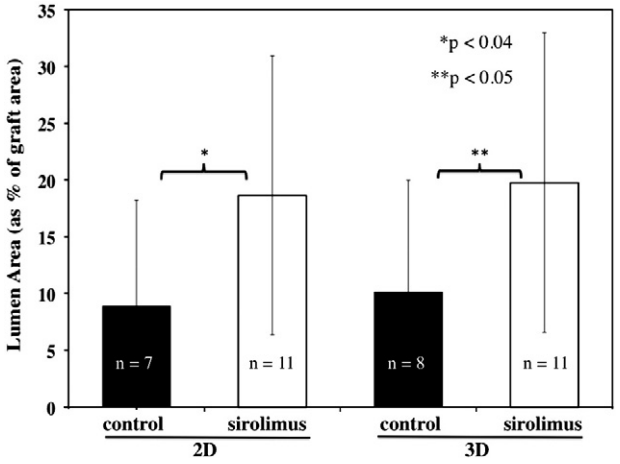


**Fig. 7.** Perivascular treatment with sirolimus is associated with increased cross-sectional lumen area at the venous anastomosis. The venous anastomoses in sirolimus-treated grafts and control grafts underwent both 2D and 3D black-blood MR imaging. Each bar represents mean ± SD.

Morphometric analysis on histology slides is the most common method for the quantification of neointimal hyperplasia. It is, however, labor-intensive and requires explantation of the vascular tissues, thus prohibiting the serial monitoring of the hyperplasia over time. Histological analysis can also be complicated by artifacts, such as shrinkage of the vascular tissues that often occurs immediately after explanation and with formalin fixation. For the assessment of hyperplasia associated with ePFTE grafts, there is an additional problem because the large difference in consistency of the ePTFE material and native vascular wall makes sectioning the tissue block technically difficult while maintaining the integrity of the components. For these reasons, we established the MRI method of imaging the AV graft *in vivo* [8]. With this technique, the vascular lumen was imaged *in vivo* without the administration of contrast, which would confer a distinct advantage in patients with end-stage renal disease who are prone to develop nephrogenic systemic fibrosis with exposure to gadolinium [9]. The minimum lumen cross-sectional area was assessed and quantified using DICOM imaging software. The minimum lumen cross-sectional area is a crucial determinant of the blood flow through the graft. Using MRI in the present study, the minimum cross-sectional lumen area at the venous anastomosis in the sirolimus-treated grafts was more than twice as large as that observed in the control grafts at the end of the six-week period. A further advantage of the MRI technique over histology is that it allows for the evaluation of the lumen geometry along the entire length of the vessel of interest.

Monitoring the localization of the administered polymer gel *in vivo* is essential for the development of this treatment strategy. Using MRI techniques, we were able to confirm that the polymer gels were administered to the target anastomotic region and remained at that location during the course of follow-up (Fig. 4). MRI was also useful in monitoring the disintegration of the administered polymer gel *in vivo*. In the present study, the MRI signal of the polymer gel decreased by up to 80% at three to four weeks after injection. In contrast, complete breakdown of polymer was observed *in vitro* by six weeks. This apparent faster degradation of the polymer gel *in vivo* than *in vitro* in the present study was compatible with the previous kinetic studies reported by Zentner et al. where a subcutaneous depot of the gel degraded by six weeks in contrast to approximately eight weeks *in vitro* [7]. Using gel permeation chromatography, they reported that the degradation of the gel *in vivo* followed the pattern, albeit accelerated, of that observed *in vitro* at 37 °C. The breakdown of the gel occurs by hydrolysis to lactic acid, glycolic acid and PEG1000. The presence of catabolic enzymes, and inflammatory cells as well as possible accumulation of acidity is a potential contributing factor to enhanced degradation *in vivo*. Degradation in a deep surgical wound site could also occur more quickly than at a site of simple subcutaneous injection as the surgical wound typically has more fluid and inflammatory cells. Recently, Kempe et al. reported the use of benchtop MRI for *in vivo* imaging of the degradation and encapsulation of subcutaneous PEG400/PLGA implants in mice over a period of seven weeks [10]. Madhu et al. monitored the degradation of a variety of subcutaneously injected depot formulations *in vivo* in rats using MRI and found that the signals of the Poloxamers 407 and 188 disappeared by 45 h [11]. To our knowledge, the present study was the first to report the tracking by MRI of a drug delivery polymer applied to a deep anatomical site or a perivascular region, and in a large animal in particular. A further advantage of this MR imaging technique is the higher resolution image of the polymer gel, compared to that provided by even contrast-enhanced 3D multi-planar computerized tomography (CT) [12].

Using a similar porcine model of AV graft stenosis, Rotmans et al. reported a significant reduction in hyperplasia development at the venous anastomosis after placement of sirolimus-eluting stents, compared to bare-metal stents or unstented controls [13]. However, stent placement required cannulation of the synthetic graft, and a high incidence of thrombosis occurred that necessitated the addition of the antiplatelet agent abciximab, in addition to aspirin and clopidogrel,



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**Table 3**
Plasma sirolimus levels and serum chemistry at baseline (pre-operative) and six weeks after grafts-placement surgery in control and treated groups.

| | Plasma sirolimus levels (ng/mL) (Number of animals) | Glucose (mg/dL) | BUN (mg/dL) | Creatinine (mg/dL) | AST (IU/L) | ALT (IU/L) | WBC ($10^3$/mL) |
|---|---|---|---|---|---|---|---|
| *Baseline* | | | | | | | |
| Control (12) | b.d. (7); NA (5) | 130 ± 26 | 8 ± 3 | 0.9 ± 0.1[a] | 25 ± 4 | 41 ± 9[b] | 13.6 ± 2.8 |
| Treated (11) | b.d. (11) | 133 ± 26 | 9 ± 3 | 1.0 ± 0.1[c] | 26 ± 7 | 51 ± 12 | 15.5 ± 2.8 |
| *Six weeks* | | | | | | | |
| Control (12) | b.d. (7); NA (4) | 167 ± 64 | 9 ± 2 | 1.3 ± 0.2 | 34 ± 13 | 53 ± 9 | 11.6 ± 2.8 |
| Treated (11) | 2.3 ng/mL; 3.6 ng/mL; 2.2 ng/mL; 2.9 ng/mL; b.d.[a] (6); NA (1) | 154 ± 44 | 8 ± 2 | 1.3 ± 0.3 | 27 ± 6 | 57 ± 7 | 12.6 ± 2.8 |

b.d.—below detection limit of 2 ng/mL.
NA—not available due to inability to collect sufficient volume of blood.
[a] $p<0.02$, baseline vs. six-week creatinine in control group.
[b] $p<0.04$, baseline vs. six-week ALT in control group.
[c] $p<0.02$, baseline vs. six week creatinine in treated group.

in order to maintain patency up to four weeks. Our perivascular drug delivery approach did not involve cannulation of the synthetic graft and no increased risk of early occlusion occurred in our study. Drug-eluting stents were designed to inhibit restenosis that is initiated by a single insult to the vessel wall, such as balloon angioplasty. In contrast, the bypass or interposition graft to the peripheral artery and the AV graft are subjected to chronic pro-proliferative stimuli as a result of highly aberrant vascular wall mechanical stress forces. The AV graft is also unique in that it suffers frequent needle trauma from cannulation for hemodialysis access. Additionally, the uremic milieu that is present in the hemodialysis population renders their vascular wall abnormal on a chronic basis; for example, nitric oxide production is subnormal. This myriad of chronic stimuli necessitates chronic suppressive therapy for neointimal hyperplasia. In the clinically used coronary drug-eluting stents, sirolimus is depleted within 90 days with the majority of the drug being released in the first 30 days [14]. Thus, drug-eluting stents may not be sufficient for long-term stenosis prevention in the AV graft. Our current approach is particularly attractive in the hemodialysis AV access setting since its superficial location (almost exclusively in the arm) allows for repeat percutaneous injection of the sustained delivery polymer to the venous anastomosis.

The sirolimus concentrations in the vessel wall were determined in a previous pharmacokinetic study [5] and have now been reformatted and presented in Table 4. Based on that earlier study, only approximately 6% of the total administered dose was accounted for in the vascular tissue at the two-week time point, which was substantially lower than the 65% release expected from the previous *in vitro* pharmacokinetic experiments. The tissue concentrations at six weeks were considerably lower than the concentrations at two weeks. An intact polymer depot could not be retrieved at the later time points; thus the amount of drug remaining in the depot could not be determined to calculate more definitive mass balance. The inability to account for all drug in the *in vivo* system is likely due to loss from metabolism, diffusion to surrounding nonvascular tissue, and/or wash out in the luminal blood stream. A tissue concentration of 248 ng/g observed in the vascular wall at six weeks is approximately equal to 271.3 nmol/L, given the molecular weight of sirolimus of 914.2 and assuming the density of the vein tissue to be 1.0 g/mL. The $IC_{50}$ of sirolimus for the inhibition of porcine venous smooth muscle cells *in vitro* is 3.4 ng/mL (3.7 nmol/L) [15]. Therefore, the tissue drug concentration achieved *in vivo* in our animal model was two to three orders of magnitude greater than the necessary concentrations required for the inhibition of cultured porcine smooth muscle cell proliferation. The ideal sirolimus amount in the polymer and application time points to achieve maximum stenosis inhibition and safety are yet to be determined. If necessary, the drug release kinetics from the polymer can be modified to achieve desired profiles by altering the molecular weight and ratio of the specific polymer components [7]. It is possible that fewer drug applications or applications spaced further apart in time would have been efficacious as well.

The systemic exposure to sirolimus in the present study, as indicated by the peripheral plasma drug concentrations, appeared to be minimal. However, there was substantial swelling with exudate at the site of drug application in three of 18 treated animals (an incidence of 16.7%) that required early termination from the study. In two of these three animals, the formulation used in the first, but not subsequent, applications included dimethylsulfoxide (DMSO), which was subsequently discontinued in our animal experiments. The swelling had not been observed in other animals in previous experiments when the identical triblock polymer alone was administered to the perivascular area. Thus, the swelling could potentially be a result of exposure of the tissues to high concentrations of sirolimus. The addition of DMSO did not appear to significantly alter the *in vitro* release rate of sirolimus. Local toxicity of sirolimus appears to be dose-dependent. Recently, Rajathurai et al. reported that approximately 25% of porcine vein-to-artery interposition grafts ruptured, typically within 5–7 days after

**Table 4**
Venous anastomotic tissue concentrations of sirolimus after graft placement in the treated group.

| Time of explants | Number of drug applications | Total amount of drug applied | Max.[a] tissue contraction of drug: ng drug/g tissue (molar concentration)[b] |
|---|---|---|---|
| One week | One (applied at time of surgery) | 5 mg | 2.4 ± 0.6 ng/g (2.6 nmol/L)[c] |
| Two weeks | Two (applied at time of surgery; injected one week after surgery) | 10 mg | 1561 ± 37 ng/mg (1707.5 nmol/L)[c] |
| Six weeks | Four (applied at time of surgery; injected one, two and three weeks after surgery) | 20 mg | 248 ± 13 ng/mg (271.3 nmol/L)[c] |

Tissue concentrations of drug were previously reported in a different from but are included here for completeness.
[a] Maximum concentration indicates the highest amount of drug that was found within a region of vein tissue 3 cm distal or proximal to the venous anastomosis along the length of the contiguous vein at the time point indicated.
[b] Tissue used for this analysis included the venous anastomosis and ~3 cm of contiguous proximal and distal vein segments.
[c] Molarity was calculated based on the mol. weight of 914.2 g/mol and assuming that 1 $cm^3$ of tissue weighed 1 g.



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grafting, with the perivascular delivery of 120 mg of sirolimus per $cm^2$ of graft using polyvinyl alcohol microspheres suspended in pluronic gel as the delivery platform [16]. They estimated that 60% of the drug was released by four days, whereas the release of sirolimus from the triblock polymer gel in our *in vitro* study was only approximately 20% in four days [5]. Kawatsu et al. reported inhibition of hyperplasia in a canine model of femoral artery interposition grafts after wrapping with a biodegradable copolymer film embedded with up to 800 mg of sirolimus but reported no toxicity [17]. Additionally, Schachner et al. did not report any toxicity when a pluronic gel laden with 200 mg sirolimus was delivered perivascularly to inferior vena cava grafts in a mouse model [18].

In conclusion, perivascular delivery of sirolimus is an attractive means to inhibit neointimal hyperplasia development. The sustained delivery of this drug over a prolonged duration by repeat percutaneous injection of the drug-laden polymer is particularly useful under conditions of chronic stimulus, such as the AV graft used for hemodialysis.

## Disclosures

CMT, LL, HL, IZ, DKB, SEK, SCO and AKC have no relevant disclosures. K.D. Fowers and R. Rathi were (and Dr. Fowers continues to be) employed by Protherics Salt Lake City, Inc., a BTG Company, which supplied the ReGel™, at the time of this work.

## Funding sources

This work was supported by RO1HL67646 (AKC) from the National Heart, Lung and Blood Institute, a Merit Review award from the Department of Veterans Affairs (AKC) and the Dialysis Research Foundation of Utah & Idaho (CMT and LL).

## Acknowledgments

The authors acknowledge the assistance of Yuxia He in animal surgeries. Henry Buswell and Melody Johnson provided assistance in MR imaging at the Center for Advanced Medical Technology (CAMT) at the University of Utah. Rock Hadley, also of the CAMT, was instrumental in the development of the customized MR coil used in the MR imaging. Bradley Baird and the Biostatistical Resource Facility of the Center for Clinical and Translational Science at the University of Utah provided assistance in statistical analyses.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jconrel.2012.03.011.

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