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Lowering the Dose of Sirolimus, Released From a Nonpolymeric Hydroxyapatite Coated Coronary Stent, Reduces Signs of Delayed Healing

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Objectives The aim of this study was to compare efficacy of low- and high-dose sirolimus release (25, 40, or 100 μg) from hydroxyapatite (HAp) with Cypher (Cordis, Johnson & Johnson, Warren, New Jersey) (111 μg sirolimus) in porcine coronary arteries.

Background Polymer-based sirolimus-eluting stents such as Cypher interfere with vascular healing, probably due to the permanent presence of the polymer coating and the high sirolimus dose. The use of low-dose sirolimus and inert nonpolymeric but biodegradable coatings such as HAp might be more appropriate.

Methods Stents (n = 68) were implanted, guided by quantitative coronary angiography. All swine received clopidogrel and acetylsalicylic acid during 28 days follow-up. Safety of the coating in absence of drugs was studied by comparing HAp with and without a lipid-based release regulating layer (HApR) with bare-metal stents. Efficacy was studied by comparing the release of 25, 40, and 100 μg sirolimus with Cypher.

Results The safety study (without drug) revealed no differences in neointimal thickening in response to HAp and HApR with complete healing in all groups. Dose response analysis showed that neointimal thickening was similar in all groups regardless of sirolimus dose, with a normal appearance of the endothelium. There was, however, a dose-dependent increase in fibrinoid ($p = 0.028$), considered to be a marker of delayed healing. The Cypher stent induced the highest amount of fibrinoid.

Conclusions Reducing the dose of sirolimus eluting from a biocompatible HAp coated stent reduces signs of delayed vascular healing, without affecting neointimal hyperplasia. (J Am Coll Cardiol Intv 2009;2:284–90) © 2009 by the American College of Cardiology Foundation

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An important limitation of current drug-eluting stents (DES) are their association with delayed healing as well as their negative effect on endothelium-dependent vasomotor function distal to the stent, which can even extend into the microvasculature (1–5). Toxic tissue reactions to the polymer coatings and the relatively high drug levels might explain these processes. Although it remains to be determined whether delayed healing in DES is truly associated with late stent thrombosis (6,7), it is clear that higher drug doses at overlapping stent sites do affect vascular healing (8). Lower but still effective drug doses in combination with nonpolymeric coatings might be advantageous in reducing all of these phenomena.

In the present study we tested the biocompatibility and efficacy of a polymer-free base coating consisting of hydroxyapatite (HAp) with and without a drug release regulating lipid layer (HApR) eluting incremental doses of sirolimus (25, 40, and 100 μg). Outcome was compared with the commercially available polymer-based DES Cypher (Cordis, Johnson & Johnson, Warren, New Jersey). End points were neointimal formation, delayed healing, and vascular injury in a swine model of coronary stenting.

Methods

Animals. Experiments were performed in farm-bred swine (Yorkshire-Landrace, 30 to 35 kg) as previously described (9). The study complied with the regulations of the animal care committee of the Erasmus University Rotterdam and the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication 85-23).

Stents. All stents are based on a 316L stainless steel (low-magnetic, low-carbon) balloon-expandable coronary stent (length 16 mm, width 3.0 and 3.5 mm) built by MIV Therapeutics, Atlanta, Georgia. As a control, Cypher stents (Cordis, Johnson & Johnson; length 13 mm, width 3.0 mm) were used.

Coatings. The study-coatings are based on nonpolymeric HAp (Fig. 1) with a drug release regulating lipid layer (HApR) for regulating the release of higher doses of sirolimus. In vitro testing on crimped and expanded stents at 37°C in 7.4 PH buffered saline demonstrates a drug release rate (in μg drug/time point) that is almost the same as that released by Cypher during the first hour. In vitro drug release is calculated to reach 100% in approximately 3 to 4 weeks (data are on file at MIV Therapeutics). Coating characteristics are summarized in Table 1.

The Cypher stent releases sirolimus from a mixture of 2 non-erodible polymers (polyethylene-co-vinyl acetate and poly-butyl-methacrylate) that are combined (67% and 33%, respectively) and then applied to a parylene-C coated stent. A drug-free top-layer of poly-butyl-methacrylate serves to prevent a burst effect. The stent was designed to release 80% of the drug within 30 days.

DES study. The safety and efficacy of the polymer-free HAp coating was assessed by comparing HAp (n = 6) and HApR (n = 6) with HAp releasing 25 μg and HApR releasing 40 μg and 100 μg sirolimus (n = 13/dose) with Cypher containing 111 μg sirolimus (n = 13) at 28 days follow-up. All animals received 3 different stents each, 1/coronary artery, according to a predetermined randomization scheme.

Animal preparation. Animals were prepared as described earlier (10). In short, animals were pretreated with 300 mg acetylsalicylic acid (ASA) and a loading dose of 300 mg of clopidogrel (Plavix, Sanofi Aventis, Gouda, the Netherlands) 1 day before the procedure. After induction of anesthesia and connection to the ventilator, antibiotic prophylaxis was administered by an intramuscular injection of streptomycin, penicillin procaine (0.1 mg/kg). An introducer sheath was placed in the carotid artery for arterial access. A dose of 250 mg ASA and 10,000 IU heparin was administered (i.a.).

Stent implantation and angiography. Stent implantation was performed as described earlier (9,10). Under guidance of quantitative angiography (CAAS II, PIE Medical, Maastricht, the Netherlands), arterial segments of 2.5 to 3.2 mm in diameter were selected in each of the coronary arteries. Stents were placed with a balloon/artery ratio of 1.1 according to a predetermined scheme (random block design). After the final quantitative angiogram, the animal was allowed to recover from anesthesia and returned to the animal care facilities for the postoperative recovery. During the follow-up period, 300 mg ASA and 75 mg clopidogrel were administered daily.

Abbreviations and Acronyms

ASA = acetylsalicylic acid
DES = drug-eluting stent(s)
HAp = hydroxyapatite
HApR = hydroxyapatite including drug release regulating lipid layer
QCA = quantitative coronary angiography

Follow-up study. At 28 days follow-up, animals were anesthetized; quantitative angiography was repeated, followed by killing through an overdose of pentobarbital. Hearts were pressure fixed in situ with 500 ml 4% buffered formaldehyde in preparation for histology and immunocytochemistry (10).

Histology. The arteries, including 1 to 2 cm of coronary vessel both proximally and distally adjacent to the stent, were processed for plastic embedding (11,12). Sections were collected from the nonstented adjacent artery and at 3 levels (the proximal, medial, and distal part of the stent) from the stented segments and stained with hematoxylin-eosin and resorcin fuchsin (elastin stain) for quantitative and qualitative analysis.

Morphometry. Morphometric analysis, inflammatory score, and injury score analysis were performed as described earlier (13,14). Inflammatory scores were determined by the presence of inflammatory cells surrounding the stent struts, "no inflammation" being defined as no inflammatory cells being present around the struts and "full inflammation" meaning

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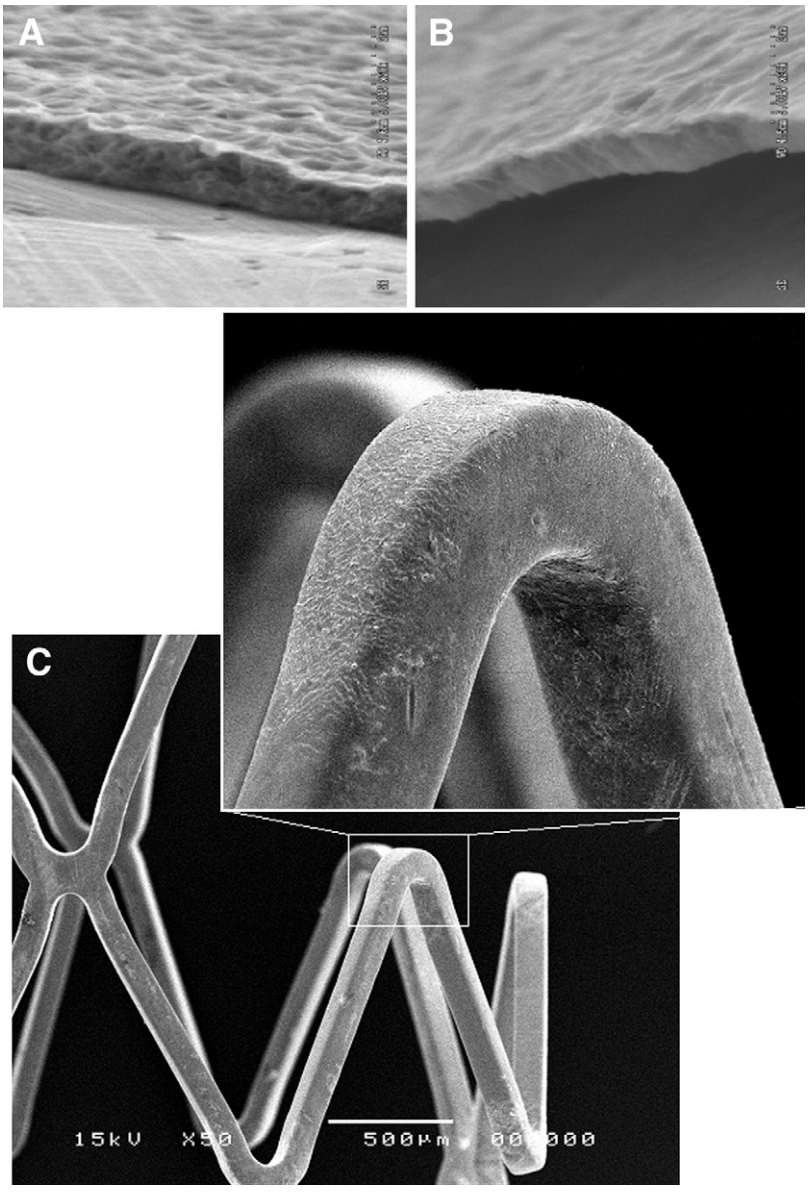

Figure 1. Scanning Electron Microscopy of the HAp Coating

Scanning electron microscopy of the hydroxyapatite (HAp) coating showing the rough surface **(A)** that is considerably smoothed by drug-loading **(B)**. The thickness of the coating is approximately 0.6 μm. The expanded HAp-coated stent **(C)** shows some roughening at areas of higher stress.

that all struts proximal, mid, and distal were surrounded by inflammatory cells.

Statistical analysis. All data are given as mean ± SD and were analyzed with SPSS (SPSS Inc. version 11.5.0, Chicago, Illinois), with the stent as the unit of analysis, not the animal.

Intergroup differences were initially assessed with analysis of variance with a post hoc Bonferroni correction. Statistical significance was considered for $p < 0.05$. Linear regression analysis was performed to take into account the effect of co-factors on outcome. These factors, to include the whole procedure, were: 1) stent characteristics (presence of HAp, release layer, and drug dose); 2) procedural characteristics (coronary diameter before stenting, stent/artery ratio, acute gain); and 3) histological characteristics (intimal inflammation, morphologic injury score). Dummy variables were included to assess differences between the study-groups. Logistic regression was performed with sirolimus dose as a variable to predict fibrinoid (best 2 tertiles vs. worst) for assessment of odds ratio and confidence intervals.

Table 1. Polymer-Free Sirolimus HAp Coating Characteristics

	Estimated Thickness	Coating Weight	Drug Amount
HAp-25	<1 μm	77 μg	25 μg
HApR-40	<1 μm	160 μg	40 μg
HApR-100	<1 μm	318 μg	100 μg

HAp = hydroxyapatite; HApR = hydroxyapatite including drug release regulating lipid layer.

Results

A total of 68 stents were successfully placed in the coronary arteries of 23 swine. Only in 1 case did an animal receive only 2 stents because of a too-small third coronary artery. All animals survived the follow-up period. At sacrifice, no macroscopic evidence of stent thrombosis or myocardial infarction was seen.

Quantitative angiography. Angiographic measurements are summarized in Table 2. Analysis of variance showed no differences in vascular diameters before or after implantation between the groups. Linear regression analysis of the data showed that acute gain was the only independent predictor of late loss—not sirolimus dose, stent/artery ratio, or coating.

Qualitative histology. Histological analyses performed at 28 days showed complete healing in HAp with the neointima containing sparse erythrocytes and only few struts inflamed (Fig. 2). The HApR also showed complete healing with few struts inflamed. However, the intimal thickening contained more extracellular matrix, and the endothelium was slightly raised.

The HAp-25 and HApR-40 showed areas that were relatively acellular with a granular appearance. These areas also contained variable amounts of fibrinoid (Fig. 3) and closely packed erythrocytes. The luminal aspect of the intima showed a more normal neointima with partly raised endothelium and adherent leucocytes. Inflammation usually coincided with a few eosinophils. Areas of abundant neointima contained abundant extracellular matrix and often vacuoles indicative of cell death.

The HApR-100 and Cypher showed a qualitative response similar to HAp-25 and HApR-40 but with more clearly defined acellular intima-media border zones and more extensive fibrinoid (Fig. 3). In case of an extensive inflammatory response, eosinophils were usually involved, extending into the luminal neointima (Cypher).

Inflammation. Full inflammation (inflammatory cells surrounding all struts, proximal, medial, and distal) was not observed in any HAp-containing stent. Partial inflammation (no more than a few struts associated with inflammation) occurred in 3 of 13 HAp-25 stents, 3 of 13 HApR-40 stents, 5 of 12 HApR-100 stents, and 2 of 12 Cypher stents. In only 1 case (Fig. 2) were all mid and distal stent struts surrounded by inflammatory cells (Cypher).

Morphometric analysis. Morphometric analysis is summarized in Table 3. Regression analysis showed that stent/artery ratio, release layer, and morphologic injury score were independent predictors of intimal thickness at the site of the stent struts ($p = 0.009$, $p = 0.045$, and $p = 0.001$, respectively). Dummy variable analysis showed that HAp-25 resulted in significantly less thickness of neointima than HAp and HApR ($p = 0.02$ and $p = 0.01$, respectively). Assessment of neointimal area showed that only morphologic injury score was an independent predictor—not stent/artery ratio. There was a trend for the release layer ($p = 0.056$), HApR to show significantly more neointimal area than all DES.

Fibrinoid was observed in all groups. Regression analysis showed that sirolimus dose was the only independent predictor for the accumulation of fibrinoid ($p = 0.028$). Although HAp showed less fibrinoid than all DES ($p < 0.012$), the low-dose HAp-25 showed significantly less fibrinoid than Cypher ($p = 0.005$). Logistic regression showed an odds ratio of 33 (confidence interval: 3 to 374) for presence of fibrinoid with the highest sirolimus dose as compared with the reference group (no sirolimus).

Morphologic injury score, traditionally viewed as the result of mechanical injury, was not predicted by the angiographic parameters; only sirolimus dose and intimal inflammation were independent predictors for injury.

Table 2. Angiographic Analysis Showing the Angiographic Measurements Performed During Stent Implantation at Follow-Up

	Before	After	Follow-Up	Late Loss	Stent/Artery Ratio
HAp	2.73 ± 0.19	2.79 ± 0.28	2.48 ± 0.33	0.30 ± 0.49	1.10 ± 0.09
HApR	2.81 ± 0.24	2.80 ± 0.26	2.38 ± 0.34	0.43 ± 0.19	1.03 ± 0.03
HAp-25	2.69 ± 0.27	2.80 ± 0.25	2.57 ± 0.19	0.23 ± 0.21*	1.09 ± 0.08
HApR-40	2.75 ± 0.17	2.85 ± 0.19	2.50 ± 0.25	0.34 ± 0.20	1.09 ± 0.04
HApR-100	2.74 ± 0.22	2.78 ± 0.24	2.45 ± 0.39	0.33 ± 0.33	1.10 ± 0.03
Cypher	2.67 ± 0.20	2.76 ± 0.20	2.43 ± 0.27	0.32 ± 0.24	1.08 ± 0.05
ANOVA	p = 0.80	p = 0.96	p = 0.77	p = 0.79	p = 0.27

*Regression analysis shows that acute gain is the only independent predictor of late loss ($p = 0.001$), not sirolimus dose, stent/artery ratio or coating. Regression analysis including dummy variables shows that only hydroxyapatite (HAp)-25 and hydroxyapatite including drug release regulating lipid layer (HApR) differ significantly ($p = 0.023$).

ANOVA = analysis of variance.

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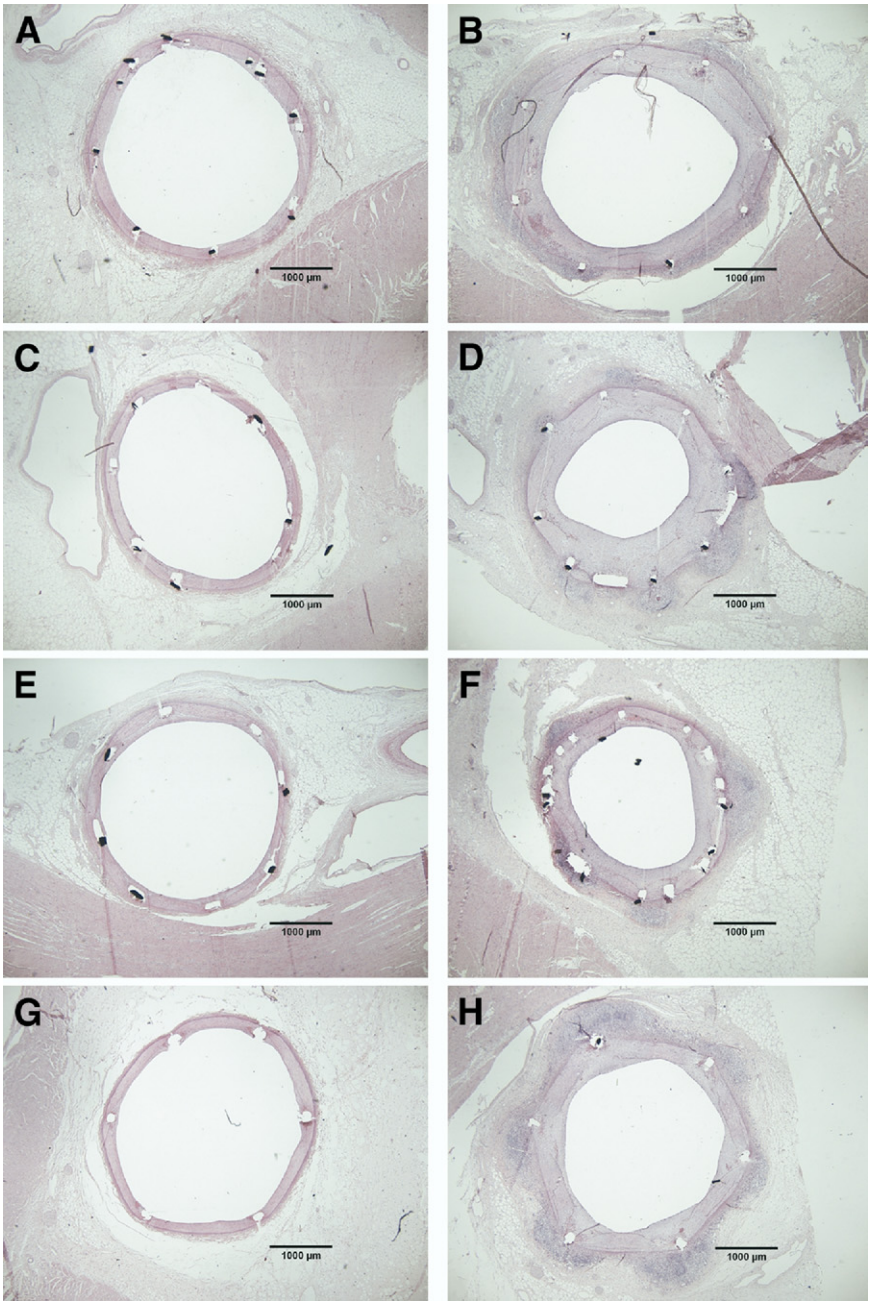

Figure 2. Histology of Sirolimus-Eluting Stents

The best and worst examples of the hydroxyapatite-coated stents loaded with 25 μg **(A, B)** 40 μg **(C, D)**, and 100 μg sirolimus **(E, F)** and Cypher **(G, H)** (hematoxylin-eosin stain).

Discussion

An important limitation of current DES are their association with delayed healing and negative effects on endothelium-dependent vasomotor function distal to the stent, which can even extend into the microvasculature (1–3,5). Because effects on vasomotor function are less or absent after bare metal stents, the drugs and the nondegradable polymer reservoirs are held responsible for these phenomena. Therefore, lower but still-effective drug doses in combination with nonpolymeric coatings might be less harmful for the recipient vasculature.

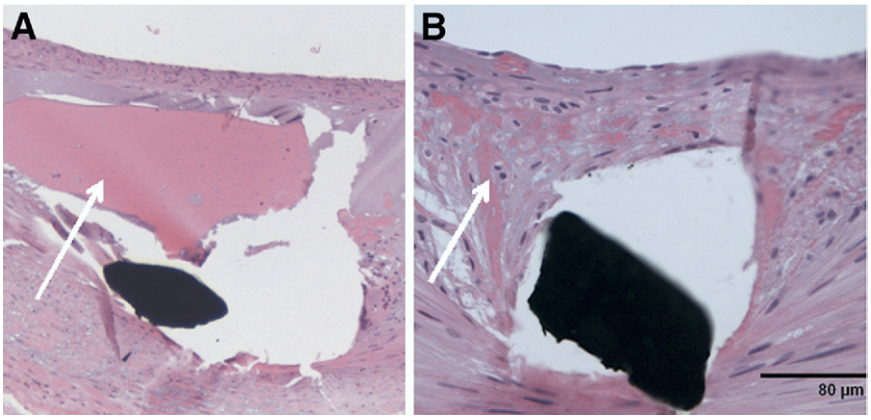

Figure 3. Fibrinoid in High- and Medium-Dose Sirolimus Stents

Detail of stent struts surrounded by fibrinoid **(arrow)**. The amount of fibrinoid in Cypher **(A)** is considerably larger than in the intermediate dose at 40 μg sirolimus **(B)** (hematoxylin-eosin stain).

The main finding of the present study is that less sirolimus, released from nonpolymeric HAp, results in less fibrinoid as a marker of vascular healing without increasing the resulting neointima formation, as compared with Cypher.

Sirolimus, delayed healing, and preservation of lumen patency. Sirolimus is known for inducing fibrinoid vascular necrosis (15,16), which is thought to be a toxic effect. We have observed an increase in fibrinoid in human atherectomy specimen from DES restenosis as late as 2 years after stenting (4). This indicates the persistence of an incomplete healing response. We postulate that a reduction in fibrinoid denotes less local toxicity, thereby indicating a faster healing response. The REDOX (Reduced Sirolimus Doses on the Bx Velocity Stent) trial showed that lower doses of sirolimus effectively maintain luminal patency for at least 12 months follow-up (17). This highlights the feasibility of lowering sirolimus dose without compromising efficacy.

Biocompatibility of the HAp coating. Hydroxyapatite, a calcium phosphate salt, is the main mineral component of bone. Because it is a naturally occurring material, it is potentially an ideal biomaterial. Handling of HAp for the purpose of coating coronary stents is difficult, however, because the material can crack or flake off during stent expansion. Particle debris in general can induce inflammation and obscure the antiproliferative effects of drugs (18,19), whereas HAp particles of 1 to 2 μm can activate macrophages to secrete tumor necrosis factor-α (20). The current coating of HAp did not induce extensive fibrosis or an inflammatory response, indicating its stability during implantation. Because late HAp resorption by osteoclasts in bone is not proinflammatory, it is not expected to induce late inflammation in the vasculature (21).

Study limitations. To study vascular healing, it is desirable to include several time points. Our study includes only 1, 28 days, the time point of largest reduction in neointimal thickness in animals using Cypher (22). This reduction is

Table 3. Morphometric Analysis: NIT Was Measured on Resorcin-Fuchsin-Stained Sections Taken From the Proximal, Medial, and Distal Region of the Stent

	HAp	HApR	HAp-25	HApR-40	HApR-100	Cypher	1-Way ANOVA p Values
NIT (μm)	282 ± 72	335 ± 49	235 ± 93	257 ± 110	239 ± 79	273 ± 90	NS
Area (mm^2)	1.75 ± 0.8	2.36 ± 0.9	1.34 ± 0.8	1.38 ± 0.7	1.43 ± 0.5	1.41 ± 0.6	0.056
F (mm^2)	0.007 ± 0.008	0.01 ± 0.01	0.05 ± 0.05	0.07 ± 0.04	0.07 ± 0.03	0.10 ± 0.06	<0.001
Injury score	0.29 ± 0.23	0.41 ± 0.29	0.27 ± 0.53	0.24 ± 0.3	0.23 ± 0.35	0.41 ± 0.47	NS

Regression analysis showed that: 1) for thickness of neointima (NIT): stent/artery ratio, release layer, and morphologic injury score were independent predictors of intimal thickness at the site of the stent struts ($p = 0.009$, $p = 0.045$, and $p = 0.001$, respectively); HAp-25 was significantly different from HAp and HApR ($p = 0.02$ and $p = 0.01$, respectively); 2) for area: morphologic injury score was the only independent predictor of intimal area ($p < 0.001$), with a trend for release layer ($p = 0.056$); HApR was significantly different from HAp-25, HApR-40, HApR-100, and Cypher ($p = 0.002$, $p = 0.007$, $p = 0.027$, $p = 0.007$, respectively); 3) for fibrinoid (F), measured on hematoxylin-eosin stained sections: sirolimus dose was the only independent predictor of F ($p = 0.028$); HAp and HApR were significantly different from HApR-40, HApR-100, and Cypher (all $p < 0.012$), and HAp-25 was significantly different from Cypher ($p = 0.005$); and 4) for injury score: sirolimus dose and intimal inflammation were the only independent predictors for injury score ($p = 0.022$ and $p < 0.001$, respectively).

Other abbreviations as in Table 2.

not always persistent in animal models studying DES (22,23). Such a late increase in intimal thickening might well be the result of a late increase in inflammation due to the persistent presence of synthetic polymer coatings (24). Because the HAp-coated stents do not contain a polymeric coating, we did not expect a late increase in neointima thickness and inflammation. Because there is no doubt about long-term clinical benefit of the Cypher stent, later time points were not included.

Although our observation of fibrinoid stands on its own, we do not know what the long-term fate of fibrinoid is. With the lack of vasomotor studies to link fibrinoid presence to vascular dysfunction, it remains to be determined whether low-dose sirolimus will indeed attenuate vascular dysfunction in response to sirolimus in the distal vasculature.

Conclusions

Lowering the dose of sirolimus eluting from a biocompatible HAp-coated stent reduces signs of delayed vascular healing.

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Key Words: animal model ■ coronary ■ drug eluting stent ■ nonpolymeric coatings ■ PCI.