

## Comparative effects of sirolimus-eluting versus bare metal stents on neointimal hyperplasia in balloon-injured external iliac artery of rabbits

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**Abstract:** The aim of this study was to check effectiveness of drug-eluting stents for prevention of restenosis resulting from neointimal hyperplasia. Twenty-one sirolimus-eluting stents (SESs) and bare metal stents (BMSs; control) were compared by implantation into left and right balloon-injured external iliac arteries of New Zealand White rabbits, respectively. Under general anesthesia and angiography, using 3-mm coronary balloon catheters, stents were successfully deployed. Anticoagulant therapy was not given to any animal. Rabbits were allotted into three groups at 7, 14, and 21 days (7 rabbits in each group). Each stented vessel of sacrificed rabbits was subjected to a resin-embedding technique and methyl methacrylate sections were obtained using a tungsten carbide knife for staining. In histological examination, SESs showed marked reductions in almost all parameters. Morphometric results showed that arteries with SESs had a larger luminal area ( $P < 0.0001$ ), lower intimal ( $P < 0.001$ ) and medial ( $P < 0.001$ ) thickness, and lower intimal index ( $P < 0.003$ ) as opposed to arteries stented with BMSs. Luminal narrowing in SESs was 0% and reendothelialization was similar in both stented groups (90.48%). SESs showed marked reduction in nonocclusive peristut fibrin deposition versus BMSs. Thus, the SES was more effective for prevention of restenosis than the BMS in rabbit.

**Key words:** Bare metal, external iliac artery, neointimal hyperplasia, rabbits, sirolimus-eluting stent

### 1. Introduction

Cardiovascular medicine is changing rapidly with the development, testing, and introduction of new diagnostic and therapeutic techniques. Percutaneous coronary intervention with stents is the method of choice for revascularization in the management of coronary artery disease. However, restenosis is a major drawback of this method (1–4). The percutaneous implantation of bare metal stents (BMSs) is associated with a high rate of restenosis (5–7). In-stent restenosis is due to neointimal hyperplasia as a result of smooth muscle migration and proliferation as well as extracellular matrix deposition. Elastic vascular recoil and constrictive arterial remodeling also contribute. Mechanical injury of the arterial wall occurred during operation by stent. This incites an acute inflammation in the vessel wall with the release of cytokines and growth factors, which in turn induce multiple signaling pathways

to activate smooth muscle migration and proliferation (8).

Drug-eluting stents (DESs) improve the clinical outcome after percutaneous coronary intervention as one of the greatest accomplishments in cardiology (9,10). DESs loaded with antiinflammatory, antiproliferative, or prohealing drugs have reduced the rates of restenosis compared with the previously used BMSs, thereby initiating a revolution in the treatment of coronary artery disease (11,12). Preclinical testing in animal models is the mainstay of the regulatory process to determine the safety and efficacy of these stents before human use. Porcine and rabbit models are extensively evaluated as they predict human responses as the stages of healing are similar, even though the temporal response to healing is significantly prolonged in humans (13). The pathophysiology of stent is related to the histological and histomorphometric evaluation of stented arteries (14).

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The sirolimus-eluting stent (SES) is a metal stent coated with sirolimus blended with synthetic polymers. After stent implantation, sirolimus is slowly released, causing localized cytostatic inhibition of proliferation of vascular smooth muscle cells in the persistent arterial wall over a period of about 1 month. Only minimal amounts of sirolimus enter the bloodstream and these appear to be insufficient to be of clinical relevance.

The sirolimus-eluting coronary stent was approved in Europe in April 2002; in the United States, FDA approval followed in April 2003. The man feasibility studies were presented and several randomized, controlled trials have documented the profound antiproliferative effects of sirolimus, a macrolide antibiotic and potent cytostatic inhibitor of smooth muscle cell proliferation. The body of clinical evidence was increased by more complex lesions (such as in-stent restenosis, small vessels, and chronic total occlusions) and “high-risk” patients such as those with diabetes. As a result of numerous single-center and multicenter national and international studies, the safety and efficacy of sirolimus-eluting coronary stents have been subjected to close scrutiny.

The aim of this study is to determine the effectiveness of DESs for prevention of restenosis resulting from neointimal hyperplasia.

## 2. Materials and methods

### 2.1. Animals

The project was approved by the Institutional Animal Ethics Committee of Anand Veterinary College, Anand, India (Project No. 2008/Surgery/51, Dt. 22.10.2008). The animals received humane care in compliance with the principles of Laboratory Animal Care formulated by the National Research Center and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

### 2.2. Drug profile of stent

In the present experimental study, the concentration of rapamycin on stents (3 × 13 mm) was 65 µg. Total amounts of drug and polymer on the DES were 23.76% and 76.23%, respectively.

### 2.3. Procedure

In 21 New Zealand White adult rabbits (*Oryctolagus cuniculus*) of either sex, of >8 months of age with average mean body weight 1.591 kg (range: 1.0–2.411 kg), sirolimus-eluting (FlexyRap, Sahajanand Laser Technology Ltd., Gandhinagar, Gujarat, India) and bare metal (FlexyStar; Sahajanand Laser Technology Ltd.) stents were deployed in the left and right balloon-injured external iliac arteries, respectively. Rabbits were allotted to 3 groups (A, B, C) of 7 each to study the stent occlusion rates at 7 (Group A), 14 (Group B), and 21 (Group C)

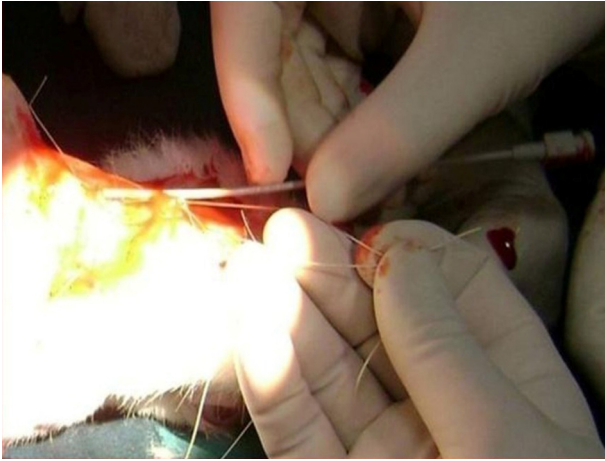
days. One day prior to the experimentation, the animals were weighed and the ventral neck region was prepared for aseptic intervention, and the diet was reduced to half on the day of operation. Rabbits were premedicated with injectable meloxicam at 0.05 mg/kg and enrofloxacin at 5 mg/kg body weight intramuscularly and anesthetized with a mixture of injectable diazepam (0.5 mg/kg), injectable ketamine HCl (40 mg/kg), and injectable xylazine HCl (2.3 mg/kg) given intramuscularly. Incremental doses were used for maintenance of anesthesia.

#### 2.3.1. Antegrade iliac artery stent implantation

Antegrade iliac artery stenting was performed as previously described (12,13,15). A small skin incision on the ventral side of the left neck was followed by separation of muscles until left carotid artery pulsation was felt. The left internal carotid artery was secured with Vicryl 3-0 threads in cranial, middle, and caudal positions (Figure 1). Two drops each of lignocaine HCl and papaverine HCl were instilled over the carotid artery. The artery was cannulated using a 24-G Jelco intra-cath guide wire and advanced under fluoroscopic imaging. After obtaining a steady hold upon the carotid artery, the cannula (5-Fr introducer sheath) was progressed in the carotid artery for iliac stenting (Figure 2). The guide wire was looped initially into the ascending aorta and then guided gently into the descending aorta. The soft-tip guide catheter and guide wire were oriented dorsally towards the common iliac artery via cranial, caudal aorta, and aortic quadrification. The left external iliac artery was wired first and a noncompliant coronary balloon angioplasty catheter of 3 mm in diameter was advanced under fluoroscopic imaging to the proximal common iliac artery, where it was inflated to 8 atm for 60 s to induce deep arterial injury. The guide wire was then advanced into the proximal portion of the right external iliac artery for balloon injury. The balloon catheter was carefully removed while the guide wire remained in place. The stent on the metallic stent platform delivery system

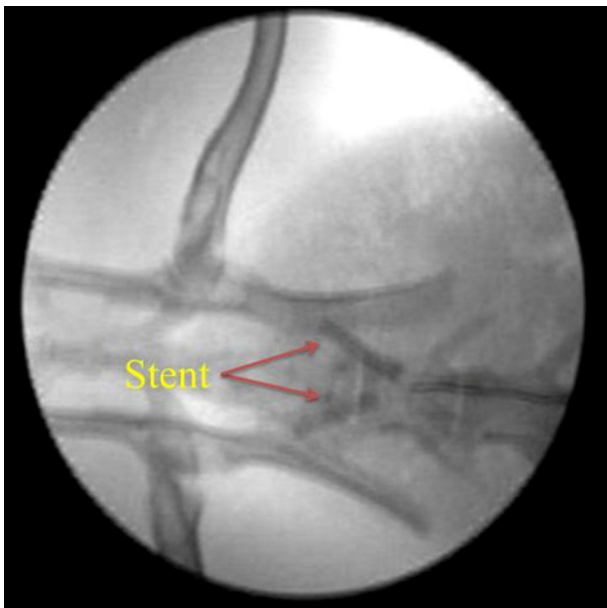


Figure 1. Triple ligation of carotid artery.



**Figure 2.** Insertion of cannula.

was advanced to the distal portion of the iliac artery using the guide wire; thereafter, the SES was deployed at the site of arterial injury. The guide wire was taken out and repositioned into the left iliac artery for BMS deployment. The BMSs were also deployed similarly at the arterial injury in the left external iliac artery. The balloon was slowly taken out under fluoroscopic imaging after deflating. Successful deployments of stents (Figure 3) were verified by fluoroscopic imaging and the stent delivery catheter was removed. The carotid artery was ligated with Vicryl 3-0 and neck musculature was sutured using an interrupted suture pattern with Vicryl 3-0 and skin with silk 2-0. Rabbits received enrofloxacin (5 mg/kg IM) and meloxicam (0.05 mg/kg IM) for 3 days after stent implantation. None of the animals were treated with anticoagulant or antiplatelet



**Figure 3.** X-ray showing stents in both iliac arteries of rabbit.

therapy after the operation. Patency of the vessels after stent implantation was determined in one rabbit from each group (7, 14, and 21 days) by a dorsal intraoperative angiography with diluted angiografin (German Remedies, Zydus Cadila Health Care Ltd.). After surgery rabbits were allowed to recover in a warm environment under a heating lamp to maintain body temperature. Green pellets and water were offered and sutures were protected with light regular antiseptic dressing.

### 2.3.2. Stent retrieval

On the day of sacrifice, animals were anesthetized with a similar protocol as mentioned for the stenting procedure and stents were retrieved. After a midline laparotomy incision to expose the stented iliac arteries, the surrounding adventitial connective tissue was separated. The descending aorta was ligated after securing access of both the femoral arteries distally. The femoral vessels after ligation were transected above and the two stented vessels specimens were separated carefully for bench dissection at the level of aortic quadrification (Figure 4). The animals were assigned to follow-up periods of 7, 14, and 21 days to be sacrificed using thiopentone sodium at 10 mg/kg body weight through a catheter fixed in the marginal ear vein.

### 2.3.3. Tissue preparation

The proximal ends of the iliac arteries were tagged with identification marks and the vessels were excised after careful separation from the adjacent fascia. Artery with stent samples were preserved in labeled vials for fixing with 10% neutral buffered formalin for at least 24 h before embedding in methyl methacrylate (MMA; E. Merck, the Netherlands). The stented external iliac arteries were dehydrated in a graded series of ethanol with three changes of MMA. The MMA-impregnated specimens were placed in glass vials and 5 mg/mL of Perkadox 16 di(4-tert butylcyclohexyl) peroxydicarbonate (Akzo Nobel



**Figure 4.** Retrieved stent.



Chemicals, India) was added to MMA as a catalyst for polymerization, and the specimens were oriented in the vials. After 1 h of vacuum impregnation to remove any air, the glass vials were carefully closed and the MMA was polymerized overnight in an oven at 37 °C. Overheating was prevented by placing the vials in a container with water. Glass vials were broken, excess MMA was trimmed off, and sections were cut on a hand-driven rotary microtome with a tungsten carbide knife (Leica, Germany). Slices of tissue were kept directly on a slide and then on a hot plate at 70 °C for 30 min. Stent cross-sections were stained with hematoxylin and eosin, Martius yellow, scarlet red and methylene blue, and Masson's trichrome stains.

#### 2.3.4. Histological and morphometric analysis

Planimetry for calculation of luminal area, intimal thickness, and medial thickness of the arterial layer was done using Microimage Lite 4.0 V (Media Cybernetics, Olympus, USA) software. For each specimen, the luminal area was obtained by direct measurement of the area outlined by the endothelium. Intimal and medial thicknesses were measured at 8 different points and the mean value was taken as the thickness. The intimal thickness was measured as the distance between the internal elastic lamina and endothelium. The medial thickness was measured as the distance between the internal and external elastic lamina. The ratio of intimal/medial thickness was used as the intimal index (16). Dimensions of the area were described in square millimeters and the intimal and medial thicknesses in millimeters. The histopathological endpoints studied by a single, blinded observer were neointimal hyperplasia, luminal narrowing,

reendothelialization, persistent inflammatory response at struts, and peristrut fibrin.

#### 2.4. Statistical analysis

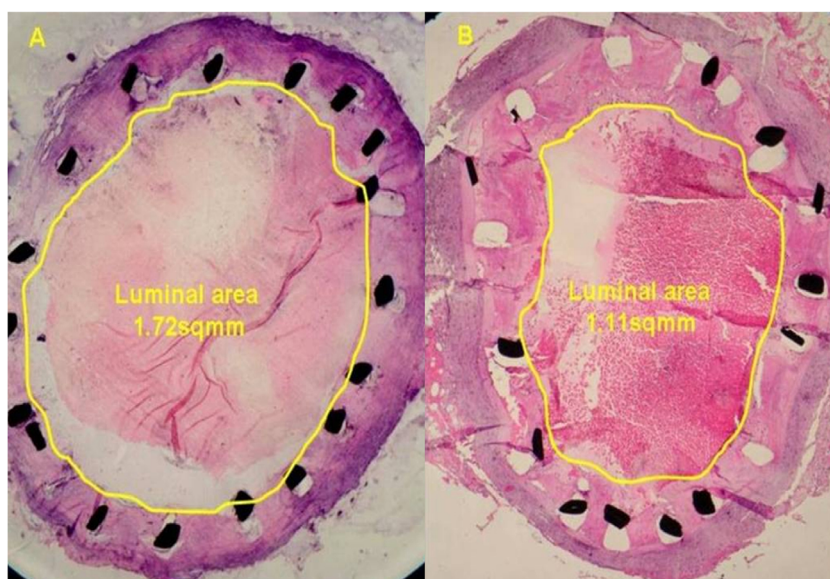
The resulting differences in the three groups of 7, 14, and 21 days were statistically compared for graded histopathological endpoints between BMS and DES. The chi-square test and Student t-test were applied to the observed data.

### 3. Results

Results were drawn from BMS and SES implantation in right or left external iliac arteries of all 21 rabbits. In one rabbit (No. 3), the SES was deployed at the posterior aorta. Local instillation of lignocaine and papaverine drops helped in desensitizing the vagus and adequately dilating the carotid artery to pass the 5-Fr introducer sheath, respectively. Hind legs of all rabbits were devoid of ischemic signs, with normal ambulation. Gait was normal in all the animals after stenting, except in one rabbit (No. 7), where mild lameness in the left hind limb 24 h after stenting was evident and persisted for 21 days. One rabbit died due to rupture of the carotid artery during stenting and was hence excluded from the study. Ligation of the posterior aorta just before branching, prior to retrieval of stented arteries, prevented loss of any fibrin or thrombus.

#### 3.1. Histopathology

SES arteries had a larger luminal area (Figure 5) of  $1.94 \pm 0.14 \text{ mm}^2$  ( $P < 0.0001$ ) and lower intimal thickness (Figure 6) of  $0.10 \pm 0.01 \text{ mm}$  ( $P = 0.001$ ) as opposed to BMS arteries. Inflammatory infiltrate (Figure 7) was present in 62% of the BMS group as compared to 42% of



**Figure 5.** Neointimal proliferation was more pronounced in bare metal stents (B) as compared to drug-eluting stents (A).

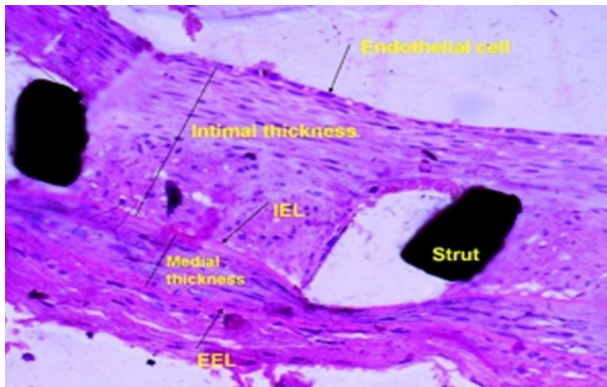


Figure 6. Intimal and medial thickness in SES.

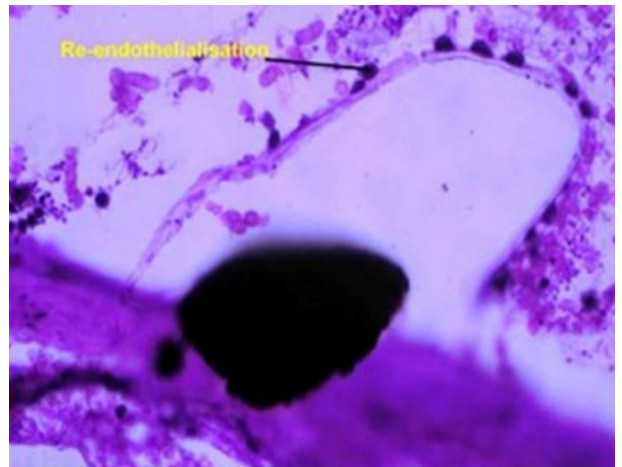


Figure 8. Reendothelialization over strut.

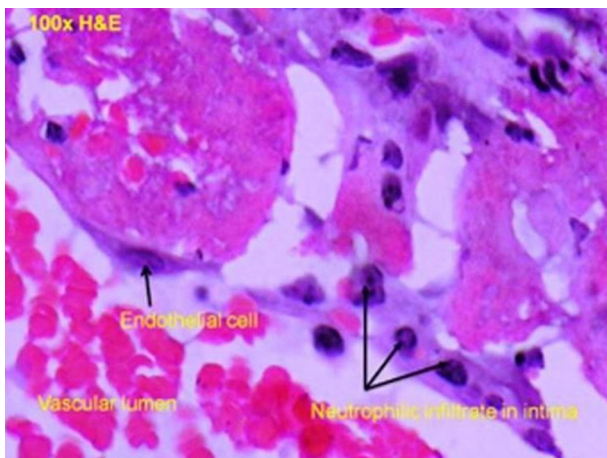


Figure 7. Inflammatory infiltration in SES.

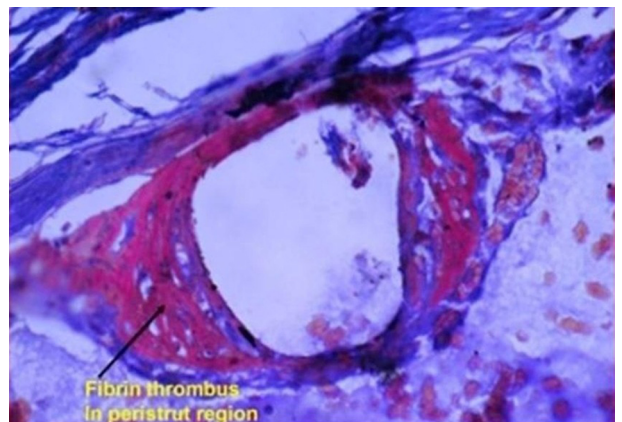


Figure 9. Fibrin thrombus in peristrut region.

the SES group ( $P = 0.01$ ). Reendothelialization (Figure 8) was present in 90% of stents in the SES as well as BMS group. There was no significant difference ( $P = 0.12$ ) in the peristrut fibrin deposition (Figure 9) between the two groups with 24% for BMS and 14% for DES (Table).

Analysis was also performed between the two groups at 7, 14, and 21 days. At 7 and 14 days there was no significant difference in any of the parameters between the two groups. At 21 days luminal area was significantly reduced in animals stented with BMSs ( $1.89 \pm 0.25 \text{ mm}^2$ ) compared to SESs ( $2.25 \pm 0.28 \text{ mm}^2$ ) ( $P < 0.01$ ).

#### 4. Discussion

This experimental study done in a rabbit iliac artery model demonstrates that SESs significantly inhibit neointimal proliferation and hence preserve arterial luminal area. The intimal thickness and intimal index were significantly increased in the BMS group as compared to the SES group, thus reducing the luminal area. In this study results are similar to those of other investigators who demonstrated that SESs inhibit neointimal proliferation

(17,18). A recent study of DESs versus BMSs in patients undergoing intracoronary bone-marrow mononuclear cell transplantation following acute myocardial infarction found a significant reduction of in-stent neointimal hyperplasia on intravascular ultrasound with DESs as compared to BMSs (19).

The mechanism by which sirolimus reduces neointimal formation has been extensively studied. Sirolimus inhibits the mammalian target of rapamycin (mTOR) and prevents the degradation of p27<sup>kip1</sup>, which is a cyclic-dependent kinase inhibitor that regulates vascular smooth muscle cell proliferation and migration. This blocks the cell cycle in the G1 (growth) phase, where RNA and proteins are synthesized. The cell is then unable to enter the S (synthesis) phase, thus preventing replication (20,21).

Furthermore, SESs showed a significant reduction in the early inflammatory response as compared to BMSs. This again confirms the findings in previously reported studies (18,22).

**Table.** Results of histomorphometry: comparison between BMS and SES.

Parameter	BMS	SES	P-value
Luminal area (mm <sup>2</sup> )	1.73 ± 0.12	1.94 ± 0.14	<0.0001
Intimal thickness (mm)	0.11 ± 0.01	0.10 ± 0.01	0.001
Intimal index (mm)	1.12 ± 0.09	1.02 ± 0.13	0.003
Inflammatory infiltrate	13/21	9/21	0.01
Reendothelialization	19/21	19/21	0.81
Fibrin thrombi	5/21	3/21	0.12

Inflammatory cells contribute to the neointimal thickening by the generation of reactive oxygen intermediates (23) and production of growth factors (24) and enzymes like cathepsins, which are capable of breaking down extracellular constituents and thereby facilitating smooth muscle cell migration (25).

Our results showed that there was no significant difference in reendothelialization between the 2 groups. Similar findings were documented in other studies (18,22). However, this observation contradicts the previously reported study where SESs showed delayed reendothelialization (26). The delayed vascular healing with SESs is due to their antiproliferative activity as a result of blocking the cell cycle at the G1 phase (20). Sirolimus also inhibits the migration and proliferation of endothelial progenitor cells, thereby contributing to the delay in reendothelialization (27). Our analysis was done at 7, 14, and 21 days. A possible explanation for this observation may be the relatively short period in which the analysis was performed.

There was no significant difference in the peristrut fibrin deposition between the two groups in this experiment. In a pooled analysis of data from four trials comparing SESs with BMSs, no significant difference was found between the two in terms of rates of stent thrombosis (28).

Finn et al. (20) reported fibrin thrombus with SESs more than 1 year after the procedure. Our analysis was over a short period and hence we may have missed out on the phenomenon of fibrin thrombus, which is associated with SESs.

Analysis done at 7 and 14 days showed no significant differences in any of the parameters between the two stented groups, but at 21 days the luminal area was

significantly reduced in animals stented with BMSs as compared to SESs.

The temporal profile of stent healing may be able to explain this observation. The early response to vascular injury due to the deployment of stents consists of the deposition of platelets/fibrin around the struts (1 to 3 days). By the seventh day, the organizing thrombi surrounding the struts contain smooth muscle cells, macrophages, lymphocytes, red blood cells, and luminal endothelial cells. By day 14, fibrin still persists along with a few chronic inflammatory cells around the struts. The neointima at this stage contains a few smooth muscle cells within a proteoglycan-rich matrix. At 28 days, the neointima contains more smooth muscle cells, proteoglycans, and type III collagen. Macrophages are rare and fibrin is usually absent. Cell proliferation is greatest at the seventh day, is reduced to approximately half at 14 days, and returns to a low baseline level by 1 month (13).

In this experimental study, the luminal area was significantly decreased in the BMS group as compared to the SES group at the 21st day. We assume that this is because the antiproliferative effects of sirolimus may be visible only by the 21st day. Results show that the SES was effective in inhibiting neointimal proliferation and luminal narrowing when compared with a BMS in a rabbit external iliac model.

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