Original Article

Effect of a Novel Stent on Re-Endothelialization, Platelet Adhesion, and Neointimal Formation

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Aim: Vascular endothelial-cadherin (VE-cadherin) is specifically expressed by outgrowth endothelial cells (OECs). Zwitterionic stent showed high antifouling and excellent blood compatibility. Therefore, we hypothesized that anti-VE-cadherin antibody-coated zwitterionic stents (VE-cad-Z stents) would promote re-endothelialization, reduce neointimal formation, and resist thrombus.

Methods: VE-cad-Z stents were examined using platelet adhesion test, platelet activation, and OEC capture ability in vitro. In vivo effect of VE-cad-Z stents on re-endothelialization, thrombus-resistance, and neointima hyperplasia was investigated in left common carotid arteries of rabbits (n=15).

Results: In vitro, VE-cad-Z stents showed better platelet-resistance and OEC-capture ability (DNA concentration: 297.23±22.71 versus 67.49±15.26 ng/µL, P<0.01). In vivo, VE-cad-Z stents exhibited better patency rate than bare metal stents (BMS) (15/15 versus 12/15), and it significantly reduced platelet adhesion and neointima formation (neointima area: 1.13±0.05 versus 1.00±0.05 mm², P<0.01 and 3.04±0.11 versus 1.05±0.06 mm², P<0.01, at 3 and 30 days, respectively; % stenosis: 20.99±0.98 versus 18.72±0.97, P<0.01 and 56.46±2.20 versus 19.45±1.24, P<0.01, at 3 and 30 days, respectively).

Conclusion: These data suggested that VE-cad-Z stents could specifically capture OECs, consequently promote endothelial healing, and also reduce platelet adhesion and neointima formation.

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Key words: Small diameter stent, Vascular endothelial-cadherin, Zwitterionic structures, Restenosis, Re-endothelialization

Introduction

Drug eluting stents (DES) have greatly reduced in-stent restenosis (ISR) rate of small diameter disease. However, these anti-healing stents interfere with the process of natural vascular healing by preventing or delaying the formation of a functional endothelial monolayer over the stents and thus cause some serious safety problems like stent thrombosis and neointimal proliferation⁵-⁷. Previous studies suggested that rapid re-endothelialization of the stented area could reduce the risk of inflammation and subsequent thrombus formation⁸. Therefore, the endothelial progenitor cells (EPCs) capture stents (Genous™ Bio-Engineered R Stent, OrbusNeich, Fort Lauderdale, Florida) coated with anti-human CD34 monoclonal antibody have been developed and shown to enhance endothelialization⁵⁻⁷. However, CD34-positive cells are capable of differentiating into different kinds of cells including inflammatory cells and vascular smooth muscle cells.

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The problem, the lack of specificity to capture EPC, may explain the increased ISR of Genous Bio-Engineered R Stent. Therefore, cell-specific molecular targeting EPC-capture without activation of platelets or recruitment of inflammatory cells and vascular SMC is important for developing more efficient and safer DES in the future.

Recently, J.M. Lee et al. carried out a head-to-head comparison between anti-CD34 antibody-coated stents and anti-VE-cadherin antibody-coated stents. The results revealed that anti-VE-cadherin antibody-coated stent could capture circulating outgrowth endothelial cells (OECs) and endothelial cells more effectively and specifically in vitro and in vivo. Moreover, VE-cadherin is specifically expressed in adherent junctions of endothelial cells and performs important functions such as intracellular signaling and cell-cell adhesion. Therefore, we hypothesized that VE-cadherin may be an ideal target surface marker for capturing EPC.

Our previous study showed that zwitterionic stent had high anti fouling and excellent blood compatibility using tests such as platelet adhesion tests, hemolysis assay, coagulation time tests. Therefore, in the present study, we applied anti-VE-cadherin antibody to our newly developed zwitterionic stents. We hypothesized that these VE-cad-Z stents may enhance re-endothelialization and resist thrombus effectively.

**Materials and Methods**

**Fabrication of VE-cad-Z Stents**

Bare metal stents (BMS) were purchased from Yinyi Co. (Dalian, China), and all chemical reagents and solvents without specific note were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). BMS-CuBr stents with atom transfer radical polymerization (ATRP) functionalities were prepared as previously described. For the preparation of polyethylene glycol methacrylate (PEGMA) brushes on the BMS-Br surface, PEGMA (8 ml, 24.6 mmol), CuBr (0.0360 g, 0.246 mmol), CuBr2 (0.0120 g, 0.0500 mmol), bpy (0.0760 g, 0.492 mmol) in 8 ml of DMF was added in a three-neck flask. After three cycles of filling and deflating gas, the reaction proceeded with a protection of argon at 35°C for 4 h. The resulting surface was rinsed using copious amounts of DMF, ethanol, and deionized water, followed by drying under reduced pressure. For the production of BMS-poly(ethylene glycol)-2-ethacryloxyethyl phosphorylcholine (BMS-PEG-MPC) stents, the reaction was carried out using CuBr (0.057 g, 0.390 mmol), bpy (0.125 g, 0.809 mmol), MPC (2.36 g, 7.99 mmol) in 7.5 ml methanol under an argon atmosphere at room temperature for 24 h. BMS-PEG-MPC was washed thoroughly using ethanol and deionized water to ensure complete removal of the physically adsorbed reactants, before drying in a vacuum desiccator under reduced pressure overnight. Finally, 10 µg of mouse monoclonal anti-human VE-cadherin antibody (ab155350, Abcam) was used in antibody adsorption after BMS-PEG-MPC in 50 ml MES buffer (0.05 mol/l, pH 5.60) reacted with 100 mg EDC and 60 mg NHS for 10 min.

**Platelet Adhesion Test in vitro**

The BMS and VE-cad-Z stents were placed into cell culture plates. To equilibrate BMS and VE-cad-Z stents, 1 mL of phosphate buffered solution (PBS) was added in each well and allowed to remain for 12 h. After removing the PBS, 1 mL of freshly prepared platelet rich plasma (PRP) of human blood, which was prepared from citrated fresh human blood, was dropped on each well and allowed to remain for 90 min at 37°C. The PRP was removed using an aspirator, and each well was rinsed three times with 1 mL of PBS. Then, 1 mL of 2.5 vol % glutaraldehyde in PBS was added in each well, and the materials were maintained at room temperature for 30 min to fix the platelets on the stents. The stents were washed with PBS again and then subsequently dehydrated by systemic immersion in a series of ethanol–water solutions [50, 60, 70, 80, 90, 95, and 100% (v/v)] for 30 min each and allowed to evaporate at room temperature. The surfaces of stents with platelets were observed using a scanning electron microscope (SEM, Model S-2260N; Hitachi Co. Ltd.). For SEM, six identical specimens were observed, and the presented images are representative.

**Platelet Activation in vitro**

To measure the platelet activation, the PRP was incubated at 37°C with four types of samples. The incubation mixture was removed at 30 min to assess the activation state of the platelets by flow cytometry. Expression of the fluorescently labeled platelet activation marker anti-CD62P and platelet pan-marker anti-CD42a was detected using a BD FACSCalibur (BD Biosciences, USA). All the platelet activation experiments were conducted in triplicate.

**Preparation of OECs**

OECs were prepared as previously described. In brief, human peripheral blood mononuclear cells (PBMCs) were re-suspended in culture medium after isolation. The PBMCs (1 × 10^7/well) were then immediately plated on collagen type I (BD Biosciences)-
analyzed by reading the whole absorption spectrum (220–750 nm) with NanoDrop ND-2000 and calculating DNA concentration and absorbance ratio at both 260/280 and 230/260 nm\(^{19}\). To further confirm the viability of the captured OECs, they were labeled with calcein AM and DAPI staining. For this purpose, stents were washed with 1\(\times\) PBS, pH 7.4 (Invitrogen), followed by staining with calcein AM (2 \(\mu\)M) (Invitrogen) for 45 min at room temperature. After the removal of free calcein by washing with Hank’s balanced salt solution (HBSS), the staining facilitated observation of live cells. Thereafter, cells were fixed with 4\% PBS-buffered paraformaldehyde (15 min), washed twice in HBSS, and stained with 406-diamino-2-phenylindole (DAPI, 0.05 mg/ml HBSS, Sigma) for 15 min to observe the nuclear morphology.

**Evaluation of OECs Capture in vitro**

The dynamic culture was followed as previously reported\(^{18}\). In brief, 10 BMS and 10 VE-cad-Z stents were connected to a circulatory loop system, which consisted of a roller pump (Zhisun Instrument Co., Ltd.) upstream of the graft and an outflow reservoir downstream of the graft. The stent was tied to the circulatory loop, and the circulatory loop was filled with culture medium (\(2 \times 10^6\) OECs/ml). Medium flowed from the roller pump through the stent to the outflow reservoir. Medium in the outflow reservoir was pumped up using the roller pump and recirculated. To simulate physiological flow rates, the pump setting was 70 strokes/min, which yielded a flow rate of 60 mL/min. At this flow rate, the calculated shear stress at the graft was 30 dyn/cm\(^2\). The entire system was installed in an incubator at 37°C in a humidified environment with 5% CO\(_2\). After 24 h of perfusion, the stents were retrieved for SEM observation.

DNA quantification was used to estimate the OEC-capture ability of BMS and DES. The QIAmp AllPrep DNA mini kit (Qiagen, Milan, Italy) was used for DNA preparation. DNA quantity and quality was analyzed by reading the whole absorption spectrum (220–750 nm) with NanoDrop ND-2000 and calculating DNA concentration and absorbance ratio at both 260/280 and 230/260 nm. To further confirm the viability of the captured OECs, they were labeled with calcein AM and DAPI staining. For this purpose, stents were washed with 1\(\times\) PBS, pH 7.4 (Invitrogen), followed by staining with calcein AM (2 \(\mu\)M) (Invitrogen) for 45 min at room temperature. After the removal of free calcein by washing with Hank’s balanced salt solution (HBSS), the staining facilitated observation of live cells. Thereafter, cells were fixed with 4\% PBS-buffered paraformaldehyde (15 min), washed twice in HBSS, and stained with 406-diamino-2-phenylindole (DAPI, 0.05 mg/ml HBSS, Sigma) for 15 min to observe the nuclear morphology.

**Surgical Implantation in vivo**

All care and handling of the animals were provided according to the Guide for the care and use of Laboratory Animals approved by the Ethical Committee of Researches of Nanjing University.

In brief, 30 male rabbits (New Zealand white) with a body weight between 3.5 and 4.0 kg were randomly implanted with BMS (\(n=15\)) and VE-cad-Z stents (\(n=15\)) in the left carotid arteries and were followed for 3 and 30 days. Anesthesia was induced with an injection of intramuscular ketamine (30 mg/kg) and intravenous pentobarbital (30 mg/kg) and then maintained using isoflurane and oxygen. Through a longitudinal left neck incision, left common carotid artery exposed. Before arterial clamping, heparin (100 U/kg) was administered intravenously. Then, a BMS or VE-cad-Z stent was implanted in the left common
carotid artery. Both stents had a nominal diameter of 2.5 mm and a length of 20 mm. The closure was sutured by layers. All animals were administered 40 mg aspirin in combination with 75 mg clopidogrel daily per os postoperatively. The implanted stent patency was monitored using a handle Doppler probe (HP Sonos 4500; Philips) every 3 days, and an angiograph was performed before they were harvested.

**Morphologic Study After Explantation**

Three days after implantation, five BMS and five VE-cad-Z stents were explanted for histological examination, and the remaining stents were explanted after 30 days of implantation. Midportion segments of the stents were fixed with 10% (vol/vol) buffered formaldehyde solution and dehydrated with a series of ethanol. The samples were embedded in resin and cut using a tungsten blade. The degree of neointimal growth was analyzed in the sections stained with hematoxylin and eosin. The neointimal area (NA) and the area within the internal elastic lamina (IEL) were measured by computerized morphometry, which was carried out by a single observer who was blinded to the experimental protocol. All images were captured by an Olympus XI 70 microscope equipped with an Olympus Magna Fire digital camera and were analyzed using a computerized digital image-analysis system (Image-Pro Plus version 6.0, MediaCybernetics, Silver Spring, Maryland).

SEM studies were conducted using tissues processed as previously described. After harvest, the stents were dissected longitudinally to evaluate the luminal surface as enface, fixed in 2.5% glutaraldehyde, and processed for scanning electron microscopy for evaluation of re-endothelialization and platelet resistance. Samples were incubated with HRP-conjugated Polyclonal anti-von Willebrand factor (vWF) antibody (DakoCytomation), followed by visualization with diaminobenzidine (Sigma) to characterize the endothelium of the stents.

**Western Blot Analysis**

Vessel wall expression of proteins such as endothelial nitric oxide synthase (eNOS), α-smooth muscle actin (α-SM Actin), and monocyte chemoattractant protein-1 (MCP-1) was determined using western blot analysis. In brief, the stent struts were carefully removed from the coronary arteries after harvest. Then, protein extracts (50 µg) were size fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Positive expression was analyzed using antibodies against eNOS (abcam, ab95254), α-SM Actin (abcam, ab7817), and

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![Fig. 2. Characterization of the adhered platelets on VE-cad-Z stents and BMS.](image)

A. The representative figure of FACS data to assess the proportion of the activated platelet on the negative control. B, The representative figure of FACS data to assess the proportion of the activated platelet on BMS. C, The representative figure of FACS data to assess the proportion of the activated platelet on VE-cad-Z stents. The expression of the platelet activation marker anti-CD62P-FITC on platelets was defined as % of platelet activation.
mean ± SD. Statistical analysis of differences between two groups was performed using unpaired t-test, and the statistical analysis of differences among three or more groups was assessed using ANOVA and multiple comparison tests. A p-value of less than 0.05 was considered statistically significant.

Results

Platelet Adhesion Test *in vitro*

To assess preliminary blood compatibility, the platelet adhesion on BMS and VE-cad-Z stents was evaluated by SEM observation. The surfaces of BMS were porous. After contact with PRP for 90 min, the MCP-1 (abcam, ab18678). Horseradish peroxidase-conjugated donkey anti-rabbit antibody (Santa Cruz Biotechnology) or rabbit anti-mouse antibody (Santa Cruz Biotechnology) were used as the secondary antibody at a dilution of 1:2000. Antibody binding was detected on X-ray films using an enhanced chemiluminescence method. β-actin on the same membranes was used as a constitutive marker. Quantity One software (Bio-Rad) was used to determine the results of the densities.

Statistical Analysis

Data were analyzed using SPSS 21.0 (IBM Corp., Armonk, USA). Quantitative data were expressed as mean ± SD. Statistical analysis of differences between two groups was performed using unpaired t-test, and the statistical analysis of differences among three or more groups was assessed using ANOVA and multiple comparison tests. A p-value of less than 0.05 was considered statistically significant.
OECs Capture in vitro

OECs capture ability of VE-cad-Z stents was examined by SEM, DNA quantification, and immunofluorescent analysis. After 24 h dynamic culture with OECs, few OECs attached on the BMS and aligned themselves disorderly (Fig. 3A). The cells on VE-cad-Z stents spread out completely and possessed a sustainable growth capability. OECs aligned well-organized and adhered tightly to each other to maintain the high integrity of their confluent monolayer structure, which was similar to that of the native artery (Fig. 3B). DNA quantification demonstrated that VE-cad-Z stents captured a significantly increased number of OECs than BMS (DNA concentration: 297.23 ± 22.71 versus 67.49 ± 15.26 ng/µL, P < 0.01, n = 10, Fig. 3C), which was consistent with the SEM results. In addition, immunofluorescent analysis confirmed that, in comparison with BMS, VE-cad-Z stents captured more viable OECs as evidenced by amplified calcein AM and DAPI fluorescent staining intensity (Fig. 4).
Stent Patency

Fifteen VE-cad-Z stents and 15 BMS were implanted into the carotid arteries of the rabbit. Three BMS were observed with thrombotic occlusion within 3 days by arterial Doppler ultrasound and carotid arterial angiographies, whereas all the remaining stents maintained patency till they were harvested for histological and morphometric analysis. VE-cad-Z stents exhibited excellent patency rate in vivo (Fig. 5C and D).

Platelet Resistance and Regeneration in vivo

SEM was conducted to study in vivo effects of VE-cad-Z stents on platelet resistance and regeneration. SEM results showed the following: (1) three days after implantation, only a few OECs attached on the luminal surface of the VE-cad-Z stents, which showed almost no difference between the BMS and VE-cad-Z stents group. Regarding the platelet adhesion, there was no platelet adhered to the VE-cad-Z stents luminal surface, whereas large number of platelets were observed on the BMS luminal surface (Fig. 6A and B). (2) Thirty days after implantation, the luminal surfaces of the VE-cad-Z stents were covered with a confluent layer of cobblestone-like cells orienting in a par-
Neointimal Hyperplasia

Histological and morphometric analysis was performed to access the neointimal hyperplasia. Histological analysis demonstrated the following: (1) compared with VE-cad-Z stents, a significant in-stent neointima was formed at BMS stent sites 3 days after implantation (Fig. 8A and B) (2) compared with 3 days after implantation, the neointima area of BMS increased significantly 30 days after implantation, whereas VE-cad-Z stents showed almost the same level (Fig. 8C and D). Quantitative analysis demonstrated a significant reduction in neointima formation and stenosis at the stent site of the VE-cad-Z stents (neointima area: 1.13 ± 0.05 versus 1.00 ± 0.05 mm² and 3.04 ± 0.11 versus 1.05 ± 0.06 mm² at 3 and 30 days, respectively; % stenosis: 20.99 ± 0.98 versus 18.72 ± 0.97 and 56.46 ± 2.20 versus 19.45 ± 1.24 at 3 and 30 days, respectively, Fig. 8E and F). Neointima formation and stenosis significantly increased 30 days after implantation compared with 3 days after implantation (neointima area: 1.13 ± 0.05 versus 3.04 ± 0.11 mm², % stenosis: 20.99 ± 0.98 versus 56.46 ± 2.20, Fig. 8E and F).

Protein Expression of the Stent

Densitometric analysis of Western blotting demonstrated that the expression of α-SM Actin and...
higher proliferative potential than early EPCs and marked similarity to completely differentiated ECs with regard to cellular morphology, marker expression, and the potential to form capillary-like structures21). VE-cadherin is a surface marker, which is exclusively expressed on the OECs and endothelial cells, but not on the early EPC and other leukocytes22). In our previous reports, flow cytometry analysis demonstrated that 95.8% OECs expressed VE-cadherin on their surfaces17). Therefore, VE-cadherin may be a more ideal target surface marker for capturing EPC than CD3413, 23). Biocompatible materials, containing zwitterion and PEG, have been identified as two important synthetic nonthrombogenic materials for DES 24, 25) . PEG has received much attention because of its properties of inertness for protein adsorption and cell adhesion, steric stabilization effect, and high chain mobility and low interfacial free energy26). MCP-1 in VE-cad-Z stents was significantly lower compared with BMS (α-SM Actin: 0.51 ± 0.09 versus 1.50 ± 0.15 and MCP-1: 0.14 ± 0.05 versus 2.30 ± 0.23, Fig. 9B). α-SM Actin in VE-cad-Z stents showed almost the same level compared with the native carotid artery (NCA), but MCP-1 was still significantly higher than that in NCA (α-SM Actin: 0.51 ± 0.09 versus 0.47 ± 0.06 and MCP-1: 0.14 ± 0.05 versus 0.06 ± 0.03, Fig. 9B). eNOS protein expressed in the VE-cad-Z stents was significantly higher than that in the BMS (0.49 ± 0.08 versus 0.09 ± 0.02, Fig. 9B) and showed almost the same level compared with NCA (0.49 ± 0.08 versus 0.50 ± 0.06, Fig. 9B).

Discussion

OECs are considered to be a good candidate for vascular regenerating cell therapy, as they have much

![Fig. 7. Immunofluorescent staining of the stents retrieved after implantation. A-B, Representative images of von Willebrand factor (vWF)-positive cells (green) on VE-cad-Z stents (Fig. 7A) and BMS (Fig. 7B) 3 days after implantation. C-D, Representative images of von Willebrand factor (vWF)-positive cells (green) on VE-cad-Z stents (Fig. 7C) and BMS (Fig. 7D) 30 days after implantation. Bar = 0.5 mm.](image)
and sulfobetaine chemistries and are effective in resisting thrombosis\textsuperscript{27, 28}. Therefore, we developed a new stent using anti-human VE-cadherin antibody to coat ionic structures are designed to mimic phosphatidylcholine, which is abundant in cell membranes. These structures contain phosphorylcholine, carboxybetaine, and sulfobetaine chemistries and are effective in resisting thrombosis\textsuperscript{27, 28}. Therefore, we developed a new stent using anti-human VE-cadherin antibody to coat
zwitterionic stents.

In the present study, we demonstrated the following: (1) zwitterionic polymers resisted the platelet adherence and prevented platelet activation effectively in vitro, (2) VE-cad-Z stents could capture OECs significantly in vitro, (3) anti-VE-cadherin antibody did not affect the viability or proliferation of OECs in vitro, and (4) compared with BMS, VE-cad-Z stents reduced neointimal hyperplasia and thrombus significantly 3 and 30 days after stent implantation in the rabbit model.

The thrombotic risk for DES extends far beyond that for BMS, and hypersensitivity reaction to polymers on DES causes activation of thrombotic cascade resulting in stent thrombosis. To overcome this detrimental side effect, we designed PEG-MPC zwitterionic polymers on a molecular level and anchored PEG-MPC onto the exterior surface of BMS by ATRP functionalities. In vitro, we demonstrated that the platelets adhered on VE-cad-Z stents were significantly less than that on BMS and the platelet activation of VE-cad-Z stents was markedly lower than that of BMS. We hypothesized that the seaweed-like structure on the surface of BMS possessed the excellent ability to resist protein and platelet adhesion, and PEG-MPC zwitterionic polymers could maintain the natural structure and state of the plasma protein contact it, which may enhance platelet resistance of VE-cad-Z stents.

In vitro, VE-cad-Z stents also showed excellent OEC-capture ability and biocompatibility. As a result, VE-cad-Z stent struts were covered with a confluent monolayer structure that was similar to the native artery after 24h dynamic culture with OECs. The DNA concentration of the VE-cad-Z stents group was four times more than that of the BMS group, which implied that VE-cad-Z stents captured far more OECs than BMS. Moreover, DAPI and calcein staining confirmed the excellent biocompatibility of VE-cad-ZA stents. Therefore, our present data provided strong evidence that VE-cadherin may be a promising eluting layer for promoting endothelium healing. This rapid re-endothelial ability is believed to reduce neointimal hyperplasia and thrombosis in long term outcomes.

In addition to its remarkable performance in vitro, VE-cad-Z stents showed its advantages in vivo as well. Three BMS were found occluded by thrombus within 3 days after implantation, whereas all VE-cad-Z stents remained patent until harvest. We hypothesized that both excellent biocompatibility of zwitterionic stent and impressive OEC-capture ability of VE-cadherin contributed toward reduced in vivo thrombogenicity and improved the early patency rate of VE-cad-Z stents. In vivo, VE-cad-Z stents showed excellent anti-platelet ability, whereas its ability to capture OECs was comparatively unsatisfying. VE-cad-Z stents failed to capture more OECs than BMS at 3 days after implantation. Therefore, we hypothesized that VE-cadherin antibody was not specific to rabbit OECs, and this limited its ability to capture rabbit OECs. However, after 30 days of implantation, a confluent layer of OECs covered the luminal surface of VE-cad-Z stents. We hypothesized that specific platelet-resistant structure of PEG-MPC zwitterionic polymers as described above reduced the in vivo thrombogenicity and improved the early patency rate of VE-cad-Z stents.

![Fig. 9. Protein expression of stents 30 days after implantation.](image)

A. Photographs of immunoblots of tissue from normal coronary (NCA), BMS, and VE-cad-Z stents. B. Densitometric analysis of protein expression (n=10 each). For statistical analysis, one-way ANOVA was performed. *P<0.05 between two groups, **P<0.01 between two groups. NS=not significant.
Coating VE-cadherin onto zwitterionic stents may be an attractive approach for drug-eluting stents, and VE-cadherin coated zwitterionic stents seemed capable of capturing circulating OECs and endothelial cells effectively and specifically without the activation of platelets or recruitment of inflammatory cells and vascular SMC. To the best of our knowledge, this is the first report on DES constructed by coating VE-cadherin onto zwitterionic stents, and our findings provided strong evidence for the validity of our protocol. However, this is just a preliminary study of VE-cad-Z stents, and many questions are still unanswered. Therefore, we are going to further study the long term outcome and side effect of our novel DES.

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