An Asymmetrical Dual Coating on the Stent

Prepared by Ultrasonic Atomization

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Abstract

This study aims to design an asymmetric dual coating (ADC) on the stent by ultrasonic atomization to solve the problem of delayed endothelialization and late or very late stent thrombosis which caused by drug eluting stent (DES) with symmetric coating. Chitosan loaded monoclonal platelet glycoprotein IIIa receptor antibody SZ-21 coating (CSC) was sprayed on inner surface of stents, and outer surface was sprayed CSC and poly(lactic-co-glycolic acid) (PLGA) loaded with docetaxel (DTX) coating (PDC). The coated surface was uniform without aggregation and no shedding phenomenon either before or after stent expanded. Fluorescence labeling has confirmed that the coating has an asymmetric structure. The cumulative release for SZ-21 and DTX was 40.11 % and 27.22 % within first 24 h, then DTX became the major released drug from 24 h to 7 d, after released for 28 d about 40% of the SZ-21 and 50% DTX still remained on the coated stent. It achieved that ADC can inhibit thrombosis at earlier period and inhibit vascular smooth muscle cells (VSMCs) proliferation at later period. And that ADC has good hemocompatibility and can significantly inhibit VSMCs proliferation. Finally, 4 and 12 weeks after the stent with ADC implanted into rabbit carotid arteries, it showed that the stent with ADC was safe and could effectively prevent thrombosis and in-stent restenosis.

Keywords: *Drug eluting stent; Asymmetric dual coating; Ultrasonic atomization; Thrombosis; Restenosis.*

Introduction

Coronary artery disease (CAD) has one of the highest mortality and morbidity rates in the world. The World Health Organization predicts that by 2030 the global number of deaths due to CAD will rise to 234 million ¹⁻². Percutaneous coronary intervention (PCI) has become the primary method of treatment of CAD ³, but there are still 30~50 % vascular late restenosis ⁴. With the development of vascular stents, bare metal stent (BMS) reduced the restenosis rate to 20~30 % ⁵. However, the expansion of BMS can cause intima injuries and cause various issues (e.g. thrombosis, inflammation, and in-stent restenosis (ISR)), which seriously hinders its further applications. Drug eluting stents (DES) are considered as a milestone in the history for treatment of CAD. With the application of DES, the ISR rate can be further reduced to be below 10 % ⁶. But DES can also lead to new problems, such as delayed endothelialization, late or very late stent thrombosis. The goal of this study is to design an asymmetric DES to solve these issues.

The sirolimus-eluting stent (SES) and paclitaxel-eluting stents (PES) are the first-generation DES with symmetric coating ⁷⁻⁸. However, the long-term examination has revealed that PES can reduce lesion revascularization, but there is little improvement on reduction of stent thrombosis and the mortality rate ⁹. It may even increase the stent thrombosis at late stage. So the long-term efficacy and safety of the first-generation DES has been questioned ¹⁰.

The second-generation DES, with thinner cobalt chromium alloy material and symmetric coating made by better biocompatible polymers loaded superior drugs such as everolimus and zotarolimus, has better safety and efficacy that can significantly reduce the rate of myocardial infarction and cardiovascular death ¹¹. However, there is no significant difference in the incidence of late or very late stent thrombosis ¹².

The challenge of third-generation stent is to overcome delayed endothelialization, late or very late stent thrombosis and other clinical issues. To date the candidate including biodegradable polymer-coated stents ¹³, polymer-free drug-eluting stents ¹⁴, and asymmetrical dual drug coated stents ¹⁵⁻¹⁶. One especially promising candidate is the asymmetrical dual drug coating with multiple functions, such as anti-restenosis, pro-endothelialization and anti-thrombosis. Kim et al demonstrated that a DES using spatio-temporal coating by dopamine-mediated hyaluronic acid coating (HA-DA) before asymmetric coating of sirolimus-in-poly (d, l-lactide) (P + S) can inhibit adhesion of platelets and enable good cell viability even under the existence of sirolimus. In addition, the in vivo neointima area and inflammation scores of HA-DA/(P + S) stent significantly decreased compared to those of BMS ¹⁵. Other stents with asymmetric coating and enables eluting the drug to the vessel wall (BPSES-A) reported in literature, could also potentially give faster reendothelialization after PCI and decrease ISR and late restenosis ¹⁶.

This study selected two kinds of biodegradable polymers, CS and PLGA, which are widely used as drug carrier in clinical treatment and have passed the US FDA and European Medicines Agency approval. The inner surface of the 316L stainless steel (316L SS) stent was coated with CSC while its outer surface was coated with CSC and PDC, which prepared by ultrasonic atomization. After testing the characteristics of ADC on the stents in vitro, we implanted the stents with ADC into rabbit carotid arteries to investigate intimal hyperplasia and thrombosis.

Materials and methods

Materials

316L SS stents (3.0 mm × 18 mm) were purchased from Tianjianyiliao Company, Jiangsu, China.

PLGA polymer (LA/GA:50/50, $M_w=20,000$) was obtained from Sigma Inc (St. Louis, MO, USA).

SZ-21, a mouse anti human platelet glycoprotein IIIa monoclonal antibody, was supplied by Prof. Changgeng Ruan, Jiangsu Institute of Hematology (First Affiliated Hospital of Suzhou University, Suzhou, China). CS ($M_w=600,000$, viscosity value: >400 mPa.S; CAS number: 9012-76-4) bought from Boxing Biotech Company, DTX was obtained from cooperation Pharmaceutical Company. SMCs (A7r5) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Blood was obtained from a healthy volunteer in accordance with the protocol approved by Blood Donation Law of the People's Republic of China, then anticoagulated was performed by 3.8 % (w / w) sodium citrate in the ratio of 9:1. All methods for humans blood in this study were performed in accordance with the standard ISO/DIS 10993-4 (Biological evaluation of medical devices, part 4: Selection of tests for interactions with blood) and approved by the Ethics Committee of Chongqing Medical University (Chongqing, China). New Zealand White male rabbits (2.6 to 3.5 kg) were purchased from the Experimental Animal Center of Chongqing Medical University, Chongqing, China. After one week normal feeding in the standard environment, all rabbits were randomly divided into two groups, stents with ADC (n=6) and 316L SS stents (n=6). All rabbits were fed normally pre- and post-stent implantation, 2 mg/kg aspirin and 1.5 mg/kg clopidogrel were added to the die 4 d prior before surgery.

Preparation of the ADC on the stents

The stent-spraying system consisted of four components including atomization module, gas-carrier module, solution-container module and stent-carrier module¹⁷⁻¹⁸. In this study, we used the third-generation of ultrasonic spray coated stents system CS III pro [Fig. 1].

316L SS stents were immersed in potassium dichromate solution for 15 min, and then ultrasonic

cleaning with ethanol (75 %), acetone and distilled water for 10 min, respectively. The stents were then left in vacuum oven at 40 °C for 8 h for dehydration. The coating process was summarized as below.

CSC solutions: SZ-21 (2 mg/mL) and CS (1 mg/mL) were dissolved in 1 % acetic acid. PDC solutions: DTX (1 mg/mL) and PLGA (1 mg/mL) were dissolved in methylene chloride solution. Distilled water in water tank of ultrasonic spray system was added to the liquid level height of 15 mm. 10 mL CSC solutions or PDC solutions was added into conical flask and then placed in the water tank. Open the atomization switch and carrier gas valve, regulate the pressure to 2 psi, with the stent from the carrier liquid nozzle of conical flask was 4 cm, set the parameters which after screened on the touch screen as follows: process engineering number was 15, stent spin velocity was 2 r/s and forward velocity was 1 r/s when sprayed CSC solutions, while process engineering number was 8, stent spin velocity was 3 r/s and forward velocity was 2 r/s when sprayed PDC solutions. “Process engineering number” refers to how many times the stent move back-and-forth in front of the nozzle. After CSC sprayed on the stent, the inner surface was covered by a balloon (3.0×18 mm, Boston scientific, USA) to ensure that PDC only sprayed on outer surface. The stent samples with the same treatment process were parallel visualized through a light microscope (BX-50, Olympus Co. Ltd, Tokyo, Japan) and SEM (Vega II-LMU, Tescan USA Inc, USA).

Scanning electron microscope and energy dispersive spectrometer test for the stents surface

The morphology and element content changes of each coating (316L SS group, CSC group and ADC group) after vacuum freeze dried and gold coated were determined by Scanning electron microscope (SEM) at accelerating voltages ranging from 15 to 30 kV and energy dispersive

spectrometer (EDS, INCA x-sight 7557, Oxford Instruments, UK), respectively. We also observed the surface of stent with ADC after expanded by a balloon (3.0 mm × 18 mm) at a pressure of 8 atm for 30 s (n = 3).

Fluorescence microscope test for the stents surface

Rhodamine and FITC (Invitrogen, USA) were used to label CSC and PDC, respectively. Rhodamine (1 mg/mL) was added to CSC solutions and sprayed on both inner and outer surfaces of stent. FITC (1 mg/mL) was added to PDC solutions and sprayed on the outer surface of stent. Then the ADC surface was examined by fluorescence microscope (OLYMPUS X-81, Japan) to verify the coating distribution on both surfaces of stents (n = 3).

Drug release of the asymmetric dual coating on stents

Each stents with ADC was transferred in 1 mL of phosphate-buffered saline (PBS: pH=7.4) shaken with 60 rpm/min at 37 °C. At different time interval (3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 7 d 10 d, 14 d and 28 d), the stents were put into another fresh 1 mL PBS (pH=7.4). The release of SZ-21 and DTX *in vitro* were tested by mouse immunoglobulin G (IgG) Elisa kit (MU30035, Bioswamp, China) through Multiskan Spectrum (Thermo, USA) and High-performance liquid Chromatography (Agilent 1100, USA), column ODS C18, 250 × 4.6 mm, respectively (n = 3).

In vitro hemolysis test

Anticoagulated blood sample (3.8 % sodium citrate: blood = 1 : 9) was diluted with normal saline (v/v ratio 4 : 5). Five different groups of stents (316L SS stents, stents with CPC, stents with CSC,

stents with PDC and stents with ADC) were static pre-incubated in 10 mL of normal saline for 30 minutes at 37 °C, and then 0.2 mL of diluted anticoagulated blood sample was added for another 60 minutes. After centrifuging at 1000 g for 5 min, the 545 nm OD values of supernatant was measured by Multiskan Spectrum and recorded as ODs. The normal saline was used as a negative control (ODn), and distilled water as a positive control (ODp). The hemolysis rate was calculated by the following equation (n = 3):

$$\text{Hemolysis rate (\%)} = (\text{OD}_s - \text{OD}_n) / (\text{OD}_p - \text{OD}_n) \times 100 \%$$

Platelets adhesion test

Anticoagulated blood sample was centrifuged at 1000 rpm for 10 min to prepare platelet-rich plasma (PRP). Five different stent groups were incubated in 0.5 mL PRP at 37 °C for 1 h, respectively. Washed 3 times with PBS (pH=7.4), and fixed with 2.5 % glutaraldehyde at 4 °C for 12 h. After that, the stents were dehydrated with 50 %, 60 %, 70 %, 80 %, 90 %, and 100 % ethanol for 10 min each and freeze-dried overnight. The platelets adherent to the stents were imaged by SEM at 10 kV, and average attached platelets numbers were counted randomly per 1000 × field (n = 5).

Active partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and

Fbg content test

Anticoagulated blood sample was centrifuged at 3000 rpm for 5 min to prepare platelet-poor plasma (PPP). Five different stent groups were incubated in 0.5 mL PPP at 37 °C for 30 min. 50 µl PPP was transferred to a special test tube, then 100 µl APTT reagent (Sichuan Maker Science

Technology Co, China), 100 μ l PT reagent, 100 μ l TT reagent and 100 μ l fibrinogen (Fbg) reagent were added into the tube respectively and incubated at 37 °C for 3 min. Finally, the APTT, PT, TT and Fbg content were measured by Automatic Coagulation Analyzer (CA-50, Sysmex, Japan) (n = 3).

Platelet α -granule membrane protein activation test

Five different stent groups were incubated in 0.6 mL PRP at 37 °C for 2 h, and then incubated in 0.6 mL mouse anti-human CD62P antibody solution (1 : 200 dilution in 1 % BSA) (Boster, China) for 1 h. PBS (pH=7.4) with 0.5 % Tween 20 (PBST) washed 3 times and incubated in 0.6 mL horseradish peroxidase-labeled goat anti-mouse polyclonal antibody (Bioss, China) for 1 h. PBST washed 3 times. 3,3',5,5'-Tetramethylbenzidine (TMB) color solution (Bioss, China) was added for 15 min dark reaction, then concentrated sulfuric acid was added to terminate the reaction. Finally, the 450 nm OD values were measured by Multiskan Spectrum (n = 3).

VSMCs proliferation experiments

VSMCs were cultured in DMEM/F12 and 10 % FBS with P/S under 37 °C, 5 % CO₂ condition. After 80~90 % confluence, the cells were detached by 0.25 % trypsin (Gibco, USA). Cell seeding of the stents was carried out in rotation culture equipment according to the new conditions (cell density: 2 $\times$ 10⁴ cells/mL; rotating time: 12 h; velocity: 10 rpm) that were described in previous studies ¹⁹. After 6 h, cells seeded stents were removed and put into 6-well culture plates with 2 mL complete medium, incubated for 1, 3, and 5 days. The growth and adhesion of VSMCs on the surface of the stents were monitored with a light microscope. The cells on the stents were digested

with 0.25% trypsin, and were transferred to 96-well plates. 10 μ l MTS (Promega, China) added into 96-well plates and cultured for 2 h. Finally, the 490 nm OD values were measured by Multiskan Spectrum (n = 3).

Cells seeded stents were fixed in 4 % paraformaldehyde, and then incubated with 0.1 % Triton X-100 for 10 min, blocked with 1 % BSA for 30 min. The F-actin was directly stained with FITC-Phalloidin (1:200 dilution in 1 % BSA) (Sigma, CA, USA) for 1 h, and the cell nuclei were counterstained with DAPI (Boster, China) for 5 min. The unbound molecules were washed off and the cells were imaged by fluorescence microscope (n = 3).

In vivo implantation procedures

The experimental protocol was in accordance with the “Principles of Laboratory Animal Care” (formulated by the National Society for Medical Research) and the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ethics approval was granted by Chongqing Medical University ethics review board. The rabbits were anesthetized by intraperitoneal injection of 3.5 kg/mL chloral hydrate, and 2 mL 150U heparin sodium solution (1 mg/mL) was injected into the ear vein. After isolated and exposed the carotid artery, the stents mounted on a balloon catheter were implanted into the left common carotid artery from the opening of left external carotid artery. The stent deployment conditions are less than 1.2 : 1 (stent : artery) ratio. The balloon was inflated at a pressure of 10 atm for 30 s, deflated, and then slowly withdrawn, leaving the stent in place. The animals received no anticoagulant or antiplatelet therapy after operation.

The observation of endothelialization and restenosis

Rabbits were sacrificed at 4 and 12 week (n = 3, at each time point for each group), and half of stented arteries were cut open longitudinally and fixed with 2.5 % glutaraldehyde in PBS (pH=7.4). After dehydration and critical point drying, the stented arteries coated with gold were examined by SEM at accelerating voltages ranging from 15 to 30 kV. The other arteries with stents were used for paraffin slices after the stents were completely electrolyzed with a modified stent electrolysis method ²⁰. Because rabbit carotid artery is smaller and weaker than porcine coronary artery, we adopted a low voltage (3 V) and low current method to slowly electrolyze stents to ensure tissue integrity. One part of the slices was stained by hematoxylin-eosin (HE) and used for stenosis analysis. Optical microscopy images were used for measuring external elastic membrane luminal area (EELA), internal elastic membrane luminal area (IELA), lumen area (LA) and neointimal area (NIA). Stenosis rate was obtained using a computerized morphometry system consisting of a microscope (BX-51, OLYMPUS, Japan) and a digital imaging workstation (Motic Images Advanced 3.2, MOTIC, Fujian, China), which evaluated by the following equation:

$$\text{Stenosis rate (\%)} = [1 - (\text{LA}/\text{IELA})] \times 100 \%$$

In vivo bio-safety evaluation

Myocardium, liver, spleen, lung and kidney of stented rabbits were collected and used for the pathological sections to observe the organizational structure. The samples were washed in normal saline, fixed in 4 % paraformaldehyde, embedded in paraffin, then cut, and HE stained. In addition, blood samples were taken from the central ear arteries of stented rabbits and used for blood serum biochemical detection. The blood samples of healthy rabbits were examined as control.

Statistical analysis

The data obtained in this study were reported as means $\pm$ standard deviation. Data obtained from different treatment groups were statistically compared by statistical software SPSS 11.5 (SPSS, Inc., Chicago, Illinois). To reveal differences among the groups, one-way ANOVA followed by Tukey's test was used. Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

SEM and EDS analysis of different coatings

We used SEM and EDS to analysis the surface morphology of different coatings. The surface of both CSC and ADC were smooth, uniform and without aggregation [Fig. 2(A)]. Furthermore, there was no loss, tilt and slight cracking in the bend after stent expansion [Fig. 2(C)]. 316L SS stents contains Si, K, Cr, Mn, Fe, Ni and other chemical elements, the C and O elements weight percentage was below the detection limit of EDS [Fig. 2(B)]. The C and O elements weight percentage of CSC were 17 % and 1 % respectively [Fig. 2(B)], while 25 % and 3 % in the ADC [Fig. 2(B)]. It showed that the C and O elements weight percentage was increased after sprayed PDC.

Fluorescently labeling detection verified the asymmetric of ADC

Different fluorescently labeled ADC was used to detected the asymmetric structure of coating. The CSC was stained red by rhodamine and the PDC was green by FITC. The red fluorescence intensity and green fluorescence intensity were consistent, it confirmed that the coated stents surface was uniform without aggregation. From the yellow box and blue box [Fig. 3(A, B)] can be seen that the

inner surface and outer surface were sprayed on CSC, only the outer surface sprayed on PDC.

In vitro drug release results

Within first 24 h, SZ-21 dominates in released drug and its cumulative release rate percent was 40.11 %, while DTX was 27.22 %. Till 28 d, the SZ-21 cumulative released rate percent was 64.27% and DTX cumulative released rate percent was 53.23% [Fig. 3(C)]. It was clear that the PDC only on stent vascular surface had divided the release of SZ-21 into two stages, first release about 40.00%, followed with a decrease from 24 h to 28 d. These results established that SZ-21 and DTX were released sustainably and burst-release phenomenon did not occur under the action of sustained release layers, and it achieved that ADC may inhibit thrombosis to promote endothelial repair at earlier period and inhibit SMCs proliferation at later period. In addition, the surface topographies of ADC were measured by SEM after the drug release. Before drug release, the stent surface was fully covered by drug coatings. After the drug release for 7 d, some dark areas appeared and surface roughness increased. After 28 d, larger holes appeared which was due to significant degradation of the coating in some regions [Fig. 3(D)].

Evaluation of blood compatibility *in vitro*

We assessed blood compatibility by using platelet adhesion and activation test, hemolysis rate test, APTT, PT, TT and Fbg test. A large number of platelets adhered to the 316L SS, CPC and PDC surface. In addition, some pseudopodia and aggregation appeared on the 316L SS and CPC surface. Only small number of platelets adhered to the CSC and ADC surface, the platelets were spherical and not activated [Fig. 4(A)]. The number of platelets adhered to the CSC and ADC surface were

lower than 316L SS surface [Fig. 4(B)] and could significantly reduce platelet activation level ($p < 0.05$) [Fig. 4(D)], which suggested SZ-21 on the coating could inhibit platelet adhesion and aggregation effectively. The hemolysis rates of all samples were below 1 % [Fig. 4(C)], which was far below the safe value of 5 % according to ISO 10993-4. The APTT of 316L SS stents, CPC and PDC on the stents were 37.81 s, 39.33 s, 40.65 s, and there were no significant difference. However, the CSC and ADC could significantly prolong APTT compared with the 316L SS stents [Fig. 4(E)]. Similar to the above case, the CSC and ADC could significantly prolong PT (Fig. 4F) and TT [Fig. 4(G)] and significantly reduce the content of Fbg [Fig. 4(H)] compared to the 316L SS stents.

VSMCs proliferation results

There was no significant difference in the number of VSMCs on different coatings after one day coculture. However, on 3 d and 5 d, the PDC and ADC could significantly reduce VSMCs proliferation compared to the 316L SS stents ($p < 0.05$). But the number of VSMCs on CPC and CSC was similar to that of 316L SS stents. It suggested that PDC could inhibit the proliferation of VSMCs [Fig. 5(B)]. Moreover, the immunofluorescence staining images of VSMCs also showed that the number of adherent cells on PDC and ADC were the least compared with other groups [Fig. 5(A)].

In vivo animal experiments results

4 weeks after stent implantation, the 316L SS and ADC stents surface had formed a thin layer of complete neointima. But there were blood components adhered to the endothelium on the 316 L SS stents after stent implantation 4 weeks and fibrous tissue-like material appeared after stent

implantation 12 weeks. The direction of endothelial cells was consistent with blood flow (Red arrows) [Fig. 6].

Intimal hyperplasia developed in the 316L SS stents was serious in some location after stent implantation 4 weeks, and its thickness of neointima was significantly greater than intima. The intimal hyperplasia was more serious so that vascular was a blockage in a large area, and angiogenesis appeared in the neointima 12 weeks after stent implantation. While in the stents with ADC group, there was a certain degree of intimal hyperplasia and the thickness of neointima was uniform after stent implantation 4 weeks; after stent implantation 12 weeks, the intimal hyperplasia was not uniform but its degree of intimal hyperplasia was significantly smaller than the 316L SS stents group [Fig. 7(A)].

4 weeks after stent implantation, there was no difference of EELA between the 316L SS stents and stents with ADC. However, IELA, LA and NIA of stents with ADC were significantly less than the 316L SS stents. These have demonstrated that the 316L SS stents had serious neointima hyperplasia which leading to stenosis, significantly higher than the stents with ADC [Fig. 7(B, D)]. 12 weeks after stent implantation, EELA, IELA, LA and NIA of the stents with ADC were significantly less than the 316L SS stents. The ADC can promote endometrial repair process and reduce the restenosis rate of artery contained the stents [Fig. 7(C, E)].

Evaluation of bio-safety in vivo

The blood serum biochemical detection comparison pre- and post- stent implantation is shown in Table 1 and no significant changes between each group. The morphologies and structures of myocardium, liver, spleen, lung and kidney were examined by pathological HE staining [Fig. S1].

The results showed that there were no obvious differences between healthy rabbits and stented rabbits, indicating that ADC had good biocompatibility and safety.

Discussion

First and second generation DES is usually coated on both sides of its struts. Bioactive drugs like sirolimus or paclitaxel released from the stent vascular surface could inhibit VSMCs proliferation. However, the release of drugs from the blood contact stent inner surface will inevitably affect endothelial cell adherence and differentiation, leading to delayed endothelialization and ISR ²¹. In this study, stents with ADC may be an alternative solution for this problem. Anti-proliferative drugs DTX which can significantly inhibited the proliferation of VSMCs in a concentration-dependent manner ²² are only coated on the outer surface of stents to suppress neointima hyperplasia, while antithrombotics SZ-21 which can bind platelet glycoprotein IIIa and inhibit platelet aggregation ²³ coated on inner surface of stents may inhibit thrombus formation. Since endothelium could completely cover the inner surface of stent as early as possible, it will then decrease the possibility of ISR. Furthermore, vascular dysfunction induced by vessel injury after stent implantation may be rapidly restored after endothelium repair.

In the study, the surface of asymmetric double-side drug coated stents prepared by ultrasonic atomization is smooth, uniform and no aggregation phenomenon before and after expansion. The ultrasonic atomization enables spraying small droplets, which leads to better-coated surfaces compared to other traditional mechanical methods such as pressure or gas-assisted systems. In particular, the resultant distributions of droplet size is very narrow and the droplet size can be controlled only by the ultrasonic frequency ²⁴. Our previous research has demonstrated that the SZ-

21 eluting PLLA coating prepared by ultrasonic atomization can well spread on the surface of coronary stents ¹⁷. It shows that ultrasonic atomization is a good method to prepare coated stent and our coated stents meet the requirements that can be used for subsequent animal experiments.

In the aspect of anti-thrombosis, SZ-21 is capable of inhibiting platelet aggregation and the binding of fibrinogen to the platelet in rabbit ²⁵. Therefore, SZ-21 is potentially useful for the treatment of thrombotic diseases ²⁶. The capability of SZ-21 to inhibit the binding of fibrinogen to the platelet is equivalent to that of Abciximab ²⁷.

In this study the cumulative release of SZ-21 was 40.11 % within 24 h that may inhibit acute stent thrombosis which occurred in 24 h or less. From 24 h to 28 d, SZ-21 released 24.16 % to inhibit subacute stent thrombosis which occurred between 1 d and 30 d, and about 40% of the SZ-21 still remained on the coated stent after released for 28 d, it indicated that the drug could potentially suppress late or very late thrombosis which occurred between 30 d and 1 or 2 years. So that the ADC sprayed on the stent can solve a series of side effects of hemolysis, coagulation and inflammation response which caused by plasma protein and platelet adhesion and aggregation on the stent after stent implantation ²⁸. We also found that the ADC could significantly prolong the APTT, PT and TT and reduce the amount of Fbg adsorption compare with other groups, which indicating that SZ-21 can slow down the clotting system of endogenous and exogenous system, and some studies have shown that Fbg adhesion reduction also inhibits thrombosis ²⁹⁻³¹, so the ADC have good blood compatibility. Moreover, the hemolysis rate of ADC was less than 5 % which meet the standards that clinical use of biological material requires ³².

Even though the application of CS in cardiovascular devices is limited, we still found no acute or subacute thrombotic events happen, and Shen et al fabricated a novel stent system, CS/heparin

coated lumenally and sirolimus released abluminally, no acute or subacute thrombotic events occurred after stent implantation³³. Therefore, we thought CS could still be applied to cardiovascular devices when it is combined with anticoagulants, like heparin or SZ-21.

In the other aspect of anti-proliferation, DTX cumulative release has reached 18 mg/mL (approximately 20 μ M) within 24 h and became the main release drug from 24 h to 7 d, which shows that ADC can significantly inhibit the proliferation of VSMCs. Because 10 μ M docetaxel can significantly inhibit both the proliferation and the DNA synthesis of fetal bovine serum and platelet-derived growth factor-BB-stimulated VSMCs in a concentration-dependent manner²². And in vitro cell proliferation assay, the PDC and ADC could significantly reduce VSMCs proliferation compared to the 316L SS stents after 3 d or 5 d co-culture, which indicated that DTX can potentially inhibit intimal hyperplasia and ISR caused by excessive VSMCs proliferation, migration to the intima and secretion of large amounts of extracellular matrix protein.

We use the rabbit carotid artery model to study the effectiveness of ADC inhibit thrombosis and restenosis³⁴. 4 weeks after stent implantation, the neointimal area of 316L SS stents was significantly larger than the stents with ADC, the neointima was consisted of VSMCs and extracellular matrix. Stent implantation would damage endothelial cells and release growth factors and cytokines, which lead to VSMCs migration to the intima and excessive proliferation to caused neointimal formation³⁵⁻³⁷. The stents with ADC could inhibit ISR in 4 weeks, because the DTX can destroy cells mitosis and inhibit VSMCs proliferation and migration. In addition, the effectiveness of inhibition of restenosis was verified by *in vivo* experiments. Continuous intimal hyperplasia and restenosis occurred in the 316L SS stents, while the stents with ADC had thinner neointima without excessive proliferation of VSMCs. In conclusion, the ADC can inhibit platelet adhesion, inhibit

VSMCs proliferation and migration, accelerate the stent surface endothelialization and inhibit ISR.

We also observed such a phenomenon: 4 weeks after stent implantation, endothelial cells had completely covered the surface of the stents, and the cells were rounded. With the increase of stent implantation time, the cell morphology changed and gradually changed to the spindle shape. Stent implantation will change the microenvironment of endothelial cells, which will affect its morphology and cytoskeleton ³⁸, and round endothelial cells are unhealthy ³⁹. The spindle-shaped endothelial cells can help to maintain the stability of the blood vessel environment ⁴⁰. In the straight shaped section of the stents, the endothelial cells with slabstone morphology are arranged along the strut, while the arrangement of endothelial cells became complicated in the V-shaped section of the stents, which is related to the local mechanical environment. We also found the cells arranged relatively close in the stent area, especially in the stent and the intima connected place, and become looser where gradually away from the stent area. When reached a certain distance, the arrangement of endothelial cells is no longer affected by the stent, only by the direction of blood flow, the shape of endothelial cells along the blood flow direction and elongated ⁴¹.

With CSC sprayed on dual sides of stent surface and PDC released from stent vascular surface, the asymmetrically designed stents with ADC prepared by ultrasonic atomization successfully inhibited neointima hyperplasia during 3 months implantation. Compared to the 316L SS stents, endothelialization of stents with ADC was relatively normal. What's more, no acute or subacute thrombotic events occurred. In conclusion, the asymmetrical dual drug coating on stent developed in this study are significantly better than the traditional DES, and has great potential in applying to biodegradable stent.

Conclusion:

- 1) The coating of an asymmetrical dual coating stents was relatively robust without shedding before and after balloon inflation. *In vitro* drug release has revealed that within day 1 the cumulative release for SZ-21 and DTX was 40.11% and 27.22%, respectively. After 28 days, there are still almost half of both drug retained.
- 2) The blood compatibility assessment of asymmetrical dual coating stents has demonstrated that the addition of SZ-21 can improve the anticoagulant properties of the stent. While, the addition of DTX can significantly inhibit the proliferation of smooth muscle cells. In addition, the asymmetrical dual coating stent can significantly reduce platelet activation.
- 3) For *In vivo* animal studies after 4 weeks and 12 weeks, asymmetrical dual coating stents have demonstrated to inhibit intimal hyperplasia and thrombosis as well as promote endothelialization.

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Conflict of Interest

We declare that we have no conflict of interest.

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Figure legends

FIGURE 1. The structure of ultrasonic spray system (CS III pro).

The device is divided into gas-carrier module (**a, b, c, d, e, f**), solution-container module (**g, i**), an atomization module (**h, j, k, l**) and stent-carrier module (**m, n**). Set the parameters on the touch screen, turn on the atomization switch and the carrier gas valve, atomize the coating material and spray it onto the stent. During ultrasonic spraying, the stent will move back-and-forth in front of the nozzle, while maintaining rotation, so as to evenly cover the whole surface of the stent.

a: gas cylinder; **b:** nitrogen gas; **c:** air switch; **d:** airflow; **e:** filter; **f:** catheter; **g:** water tank; **h:** Drop-In ultrasonic atomizer (JR-24, Beijing Dongfang Jinrong Ultrasonic Electrical Equipment Co. Ltd, Beijing, China); **i:** distilled water; **j:** conical flask; **k:** coating materials; **l:** nozzle; **m:** stent; **n:** console table.

FIGURE 2. The surface morphology of different stent coatings.

(A) The SEM images of surface of 316L SS stents, CSC and ADC prior to stent expansion, respectively. (B) The corresponding elemental analysis of 316L SS bare stent, CSC coated stent and ADC coated stent prior to stent expansion, respectively. (C) The SEM images of CSC coated stent and ADC coated stent after expansion.

FIGURE 3 Fluorescent labeling detection and drug release results of ADC.

(A) The stent vascular surface, V - shape section in yellow box was coated with both CSC (Red, Rhodamine) and PDC (Green, FITC). While the blood contact stent inner surface, U - shape section in blue box was coated with only CSC without PDC (Red fluorescence, no Green). (B) The cross section diagram of ADC on the stents showed the cross and coatings sprayed on the stent surface. (C) The cumulative release of SZ-21 and DTX from this coating was 40.11% and 27.22% within 24 h, respectively. Till 28 d, the SZ-21 cumulative released rate percent was 64.27% and DTX

cumulative released rate percent was 53.23%. **(D)** With the release of the drug and the degradation of the coating, the surface of the stents became rough and appeared holes (red circle).

FIGURE 4. Results of blood compatibility of different coatings surface in vitro.

(A, B) Only small number of platelets adhered to the CSC and ADC surface and not activated, which significantly lower than that of 316L SS stents. **(C)** The hemolysis ratios of all samples were well within 1%. **(D)** The CSC and ADC could significantly reduce platelet α -granule membrane protein level. **(E-H)** APTT, PT, TT and Fbg of the CSC and ADC surface were significantly higher than 316L SS stents. (*, $p < 0.05$).

FIGURE 5. The proliferation of VSMCs on different coatings.

(A) Morphology of VSMCs on different coatings. **(B)** On day 3 and day 5, the PDC and ADC could significantly reduce VSMCs proliferation compared with the 316L SS stents (*, $p < 0.05$).

FIGURE 6. SEM images of vascular samples after stent implantation.

Two group stents were formed complete neointima. Blood components adhered to the exposed surface of 316L SS stents, and fibrous tissue-like material appeared the V-shaped section of the stents (Blue box). The endothelial cells of ADC shown a cobblestone-like arrangement in the V-shaped section of the stents (Blue box) and the straight shaped section of the stents (Yellow box) and its direction was consistent with blood flow (Red arrows) after stent implantation 4 weeks and 12 weeks.

FIGURE 7. Hematoxylin and eosin staining images of vascular samples and quantitative comparison of histomorphometric analysis results after stent implantation.

(A) The stents with ADC could inhibit intimal hyperplasia compared with the 316L SS stents. The M, N, L and S indicate media, neointima, lumen and stent strut, respectively. **(B, C)** IELA, LA, NIA

of the stents with ADC was significantly less than the 316L SS stents. **(D, E)** The restenosis of stents with ADC was also significantly less than the 316L SS stents. It indicated that the ADC could inhibit neointimal hyperplasia and restenosis. * $p < 0.05$; ** $p < 0.01$.

Figure S1. HE staining images of main organs.

The main organs were no obvious differences between healthy rabbits and stented rabbits.

Fig.1

Fig.2

Fig. 3

Fig. 4

Fig.5

Fig.6

Fig. 7