ORIGINAL RESEARCH

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A new rapamycin-abluminally coated chitosan/heparin stent system accelerates early re-endothelialisation and improves anti-coagulant properties in porcine coronary artery models

Abstract

Purpose: Drug-eluting stents (DES) in percutaneous coronary intervention are more effective in preventing in-stent restenosis compared with bare metal stents (BMS); however, DES may cause late stent thrombosis, which has limited its use. In this study, the functional properties of a newly developed DES (RAP/CS/HEP), in which rapamycin was abluminally-loaded onto a chitosan/heparin coating stent (CS/HEP), were investigated in large animal artery injury models.

Methods: The effectiveness of BMS, RAP (the traditional version of rapamycin DES), CS/HEP and RAP/CS/HEP stents in preventing coagulation and promoting re-endothelialisation was examined and compared in the porcine coronary artery models with arteriovenous shunt, high load thrombus and coronary balloon injury at day 7 and 28, respectively, after stent implantation. The re-endothelialisation on these stents was further evaluated in terms of endothelial gene expression using quantitative RT-PCR.

Results: In the porcine coronary artery injury models, both RAP and RAP/CS/HEP stents were potent in reducing neointimal thickness, thus enlarging lumen area efficiently in the stented artery region compared with BMS and CS/HEP. RAP/CS/HEP stents facilitated re-endothelialisation and inhibited thrombosis more efficiently than BMS and RAP. Consistent with this, the expression of endothelial genes, such as CD31, CD34, eNOS and VEGF, was significantly elevated with RAP/CS/HEP stents compared with RAP and BMS stents.

Conclusion: Abluminal coating of rapamycin onto the endothelialisation-accelerated CS/HEP stent and may prove to be an efficient treatment for tackling the late stent thrombosis associated with the traditionally circumferential RAP stent. This new RAP/CS/HEP stent system exhibits considerably improved therapeutic activity.

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Restenosis, the recurrence of stenosis after percutaneous coronary intervention (PCI), occurs mainly due to PCI-triggered inflammation and intimal hyperplasia [1,2]. The restenosis rate has been substantially decreased by the use of bare metal stents (BMS) [3], particularly with the use of drug-eluting stents (DES) [4]; however, DES may cause late stent thrombosis, one of the major DES-linked complications [5,6,7]. A number of causes, including lack of antiplatelet therapy [8] and dysfunctional endothelium [9], potentially contribute to late stent thrombosis. Mechanistically, DES-associated delayed re-endothelialisation, which was tightly linked to dysfunctional endothelium [9,10], is one of the primary factors contributing to late stent thrombosis [11,12]; therefore, improving re-endothelialisation and subsequently preventing late stent thrombosis caused by DES remains a challenge.

One of the drugs that are used in DES is rapamycin (sirolimus). The traditional rapamycin-loaded DES (RAP) showed strong suppression of the neointimal proliferation and in-stent stenosis in both animal and human studies [13,14]. As mentioned above, RAP has been reported to cause localized hypersensitivity and late coronary thrombosis [15]. Indeed, some studies in patients showed that RAP was less efficient at promoting arterial healing compared with BMS [4,16]. The aforementioned issues associated with the use of RAP were largely attributed to rapamycin's anti-proliferative property, which leads to delayed re-endothelialisation [4].

Previously, we developed a chitosan/heparin coated stent (CS/HEP) that showed substantially improved anticoagulant properties and vascular re-endothelialisation in the arteriovenous shunt porcine model compared with BMS [17]. In the present study, we developed a new stent by coating the abluminal surface of the CS/HEP stent with rapamycin, designated as RAP/CS/HEP. We then evaluated its functional properties, in comparison with BMS, RAP and CS/HEP stents, in two porcine coronary artery models and in the high load thrombus model.

Materials and Methods

Sources of stents

The RAP (Firebird 2) was purchased from Minimally Invasive Medical Devices (Shanghai, China). The preparation of CS/HEP-coated stents was published previously [17]. To prepare RAP/CS/HEP, polylactic acid (PLA) and rapamycin (1:1) were painted onto the abluminal surface of the CS/HEP using a rolling method so that it formed an asymmetric DES at a dosage of 50 µg per cm in diameter, with the CS/HEP coat facing the vessel lumen and the PLA/rapamycin coat towards

the arterial wall. The stents were then dried at 30°C for 48 hours under reduced pressure.

Determination of time-dependent drug-release rates

The RAP/CS/HEP stents were placed in 4% BSA and tested on days 0, 1, 3, 7, 14 and 28, respectively. The coating membrane and the drug on the stents were dissolved using tetrahydrofuran, to which methanol was added to precipitate the membrane polymer. The mixture passed through a 0.2 µm pore size filter to remove the polymer precipitate, and the resulting rapamycin solution was subsequently assayed by HPLC (Acem9000, Korea) using a Kromasil C18 column (4.6 x 150 mm x 5 µl) at 55°C. The elution phase was a mixture of methanol, acetonitrile and water at ratio of 46:27:27. The flow rate was 1 ml/min, and 20 µl of sample was loaded for analysis at a wavelength of 278 nm. The release rate was calculated using the following equation: release rate = (Day 0 drug content - day x drug content) / Day 0 drug content * 100%.

Animal experiments

All animal experiments were approved by the Animal Care and Use Committee of the Shanghai University of Traditional Chinese Medicine. Guangxi pigs (25 kilogram, obtained from the Shanghai Animal Administration Centre) were fed with regular forage. For arteriovenous shunt model and coronary balloon injury model, forage included 300 mg of aspirin and 150 mg of clopidogrel daily. The animals were fasted overnight prior to experiments, 0.5 mg/kg of ketamine hydrochloride and 0.5 mg/kg of diazepam were used to sedate the animals and pentobarbital (i.p., 10 mg/kg) was used for anaesthesia before any surgical procedures.

Arteriovenous shunt model

The arteriovenous shunt model was established as previously described [18] to assess the anticoagulant activity of RAP/CS/HEP, CS/HEP, RAP and BMS. Briefly, the femoral veins and arteries of the pigs were isolated under sterile conditions, and a circuit between the arterial and venous circulation was established using disinfected extracorporeal circulation tubing containing a valve (30 cm x 3 mm). The RAP/CS/HEP, CS/HEP, RAP or BMS was expanded inside the tubing. The blood flow was maintained for 60 min at a rate of 90 ml/min. Thereafter, the tubing was sliced and the stent was removed. All of the samples were dried, weighed and compared under microscope (Olympus CKX41, Japan).

High load thrombus model

The pigs in this experiment were not treated with aspirin or clopidogrel; instead, they were only treated with 5000 U heparin during surgery. The RAP/CS/HEP, CS/HEP, RAP or BMS was implanted into the porcine coronary vessels. By analysing the changes of ST segments in the electrocardiograms, coronary angiography was performed to identify the formation of thrombus. The time of thrombus formation was recorded and compared among these different stents.

Coronary balloon injury model

Based on the coronary angiograms taken in the femoral arteries, a coronary segment with a diameter of ~3.0 mm was selected for implantation of a randomly selected stent with a size of 3.0 mm x 18 mm through a 0.014 inch steerable guide wire. The balloon was inflated for 8 seconds at an average pressure of 12 atm, achieving an approximate intended balloon:artery ratio of 1.1:1. The balloon was then slowly withdrawn and coronary angiographs were taken. Next, the catheters were slowly withdrawn, the arteriotomies were repaired, and the skin was closed. These animals received 100 mg aspirin and 100 mg clopidogrel daily following the surgery, and were assigned into four groups as follows: RAP/CS/HEP, CS/HEP, RAP and BMS. Coronary angiography was re-performed at 1 and 4 weeks of recovery.

Histology and immunohistobiochemistry

Each stent that was withdrawn on day 7 after implantation was cut into three pieces. The first piece was fixed in 10% formalin, embedded in hard resin and sectioned into 5 µm thickness, followed by hematoxylin and eosin (H&E) staining for measurement of vessel area and for further histological analysis. The second piece was fixed in 1% glutaraldehyde, freeze-dried and evaluated using a scanning electron microscope (TS 5136 MM, Tescan, Czech Republic). The third piece was fixed using immunohistochemical stationary liquid (BYT, China), followed by immunofluorescence staining against CD31 (Abcam, USA), which was analyzed under a confocal laser-scanning microscope (Leica, Germany), and quantified using Image-Pro Plus 6.0 software (MediaCybernetics, USA). The stents that were withdrawn on day 28 after implantation were also cut and then fixed in 10% formalin, embedded in hard resin, sectioned and analyzed histologically, as described above.

RNA purification and reverse transcription-quantitative PCR (RT-qPCR)

The total RNA was purified from the tissue covering the stent using the standard chloroform/ethanol method. RT was performed using PrimeScript™ RT Master Mix (Takara, Japan), followed by qPCR using SYBR® Premix Ex Taq™ (Tli RNaseH Plus, Japan) on MX3000P (Stratagene, USA). The oligo sequences used in this study are shown below: VEGF (product size: 99 bps): forward, CTTGCCTTGCTGCTC TACCTCC; reverse, GTCCATGAACCTTCAC CACTTCGTG; eNOS (product size: 128 bps): forward, CTCGTGTGAAGAACTGGGAGGTG; reverse, GGACTGTAGTTTCCGGGGCAAC; CD31 (product size: 97 bps): forward, CAAGCCTTGAGGGTCAG GAAAAC; reverse, AGCGTGAGGTTCTCCCAT TATG; CD34 (product size: 74 bps), forward, GCTCTGCTTGCTGAGTTTGCTG; reverse, TTGGCAAGGTGGAAGCAACAG; Actb (product size: 43 bps): forward, GGGCCAGAAGGACTCCTACGTG; reverse, CTCCATGTCGTCCCAAGTTGGTG.

Statistical analysis

The data were presented as mean ± standard deviation (SD). One-way ANOVA followed by the post-hoc SNK test (SPSS software) was used for multiple group comparisons. $P < 0.05$ was defined as statistically significant and $p < 0.01$ as highly significant.

FIGURE 1. In vitro determination of rapamycin release rate from the RAP/CS/HEP stents. Stents were withdrawn at designated sampling time points (day 1, 3, 7, 14 and 28, respectively) and the amount of rapamycin remaining on the stents was analyzed as described in Methods.

FIGURE 2. RAP/CS/HEP antagonizes the formation of thrombus efficiently both in vitro and in vivo. A. Representative images (20X) of the surface thrombus on BMS, RAP, CS/HEP and RAP/CS/HEP stents, all of which were flushed in the arteriovenous shunt model for 1 h. B. Both CS/HEP and RAP/CS/HEP had significantly reduced weight of thrombi compared with BMS and RAP. $**p < 0.01$ vs. BMS; $++p < 0.01$ vs. RAP. $n = 3/\text{group}$. C. Coronary arteriography was performed to identify the formation of thrombus in vivo (red arrow). D. In the high load thrombosis model on BMS, RAP, CS/HEP and RAP/CS/HEP stents, no thrombus was observed on CS/HEP and RAP/CS/HEP at the maximal observation time of 360 min. $**p < 0.01$ vs. BMS; $++p < 0.01$ vs. RAP. $n = 3/\text{group}$.

Results

Drug-releasing time course of RAP/CS/HEP

The drug-releasing rate of RAP/CS/HEP was determined at different time points in vitro. Fig. 1 shows that the release rates were $45.82 \pm 2.67\%$, $77.40 \pm 2.17\%$, $82.5 \pm 0.37\%$, $85.07 \pm 0.77\%$ and $85.42 \pm 0.66\%$ at days 1, 3, 7, 14 and 28, respectively. Thus, the majority of rapamycin was released within 7 days, and over 85% rapamycin was released by day 14. This drug release time course was comparable to that observed in the pig coronary arteries in vivo [19].

RAP/CS/HEP antagonizes thrombus formation efficiently both in vitro and in vivo

Anti-thrombotic activity is one of the basic functions required for clinically useful stents. The anti-thrombus ability of different stents was evaluated in an *in vitro* arteriovenous shunt model. The blood was flushed for 1 hour in the tubing where each of these four stents, RAP/CS/HEP, CS/HEP, RAP or

BMS, was implanted. The blood flow was blocked in all BMS- or RAP-implanted tubing in less than 1 hour, whereas it flowed smoothly for the entire hour in both RAP/CS/HEP and CS/HEP stent-implanted tubing (data not shown).

Next, all stents were withdrawn and the in-stent thrombi were evaluated. The thrombus completely covered the surface of BMS and RAP, but was hardly observed on the surface of RAP/CS/HEP or CS/HEP stent (Fig. 2A). The thrombi on different stents were then weighed. The thrombi on BMS and RAP were significantly heavier than those collected from CS/HEP and RAP/CS/HEP stents ($**p < 0.01$, vs. BMS; $++p < 0.01$, vs. RAP. $n = 3/\text{group}$) (Fig. 2B). RAP and BMS showed no significant difference in the weight of thrombi ($p > 0.05$, $n = 3$). Similarly, CS/HEP and RAP/CS/HEP showed no significant difference in the weight of thrombi ($p > 0.05$, $n = 3$). No visible thrombi were formed on either RAP/CS/HEP or CS/HEP stents following up to two hours of blood flow.

FIGURE 3. RAP/CS/HEP is efficient at preventing restenosis in porcine coronary balloon injury model. A. Representative optical photographs (25X) of the cross sectional slices of the porcine arteries with implantation of BMS, RAP, CS/HEP or RAP/CS/HEP were shown. Upper panels, after 7 days' implantation; lower panels, after 28 days' implantation. B. RAP/CS/HEP significantly suppressed neointimal thickness. * $p < 0.05$, ** $p < 0.01$, vs. BMS; \$\$ $p < 0.01$ vs. CS/HEP. C. RAP/CS/HEP significantly increased lumen area and inhibited neointimal area. Neointimal area: ** $p < 0.05$ vs. BMS; \$ $p < 0.05$ vs. CS/HEP; Lumen area: ## $p < 0.01$ vs. BMS; + $p < 0.05$ vs. CS/HEP. $n = 3/\text{group}$.

Next, the anticoagulation effectiveness of each of these four types of stents was assessed in the high thrombus load porcine model. The RAP/CS/HEP, CS/HEP, RAP or BMS stents were implanted into the porcine coronary vessels after the administration of 5000 U of heparin during the surgery, and coronary arteriography was performed to monitor the thrombus formation (Fig. 2C, red arrow). Consistent with the observation *in vitro*, thrombi were evident on the surface of the BMS and RAP stents by 60 minutes of flow, whereas no thrombi were detected on either RAP/CS/HEP or CS/HEP after the maximal length of observation time; i.e., 360 minutes (Fig. 2D. ** $p < 0.01$ vs. BMS; ++ $p < 0.01$ vs. RAP. $n = 3/\text{group}$).

Thus, CS/HEP exhibits more potency in anti-coagulation than BMS and RAP, whereas RAP/CS/HEP antagonizes the formation of thrombus as efficiently as CS/HEP both *in vitro* and *in vivo*.

RAP/CS/HEP is efficient in preventing restenosis in porcine coronary balloon injury model

The newly designed RAP/CS/HEP stents were examined to determine if they could efficiently prevent restenosis in the porcine coronary balloon injury model. Coronary angiography was used to track the restenosis on the day 7 and day 28 after implantation of BMS, RAP, CS/HEP and RAP/CS/HEP,

FIGURE 4: RAP/CS/HEP is efficient at promoting re-endothelialisation in porcine coronary balloon injury model. A. Representative SEM images of the inner wall of the stented arteries after 7 days' stent implantation are shown. B. Representative CLSM images (100X) after CD31 immunofluorescence of the inner wall of the stented arteries after 7 days' stent implantation. Lower panel, quantitative analysis of endothelial coverage was quantified using Image-Pro Plus 6.0 software based on the CD31 immunofluorescence of the inner wall of the stented artery after 7 days' stent implantation. **p<0.01 vs. BMS; ++p<0.01 vs. RAP. n=6/group. C. RT-qPCR was performed on the RNAs purified from the tissues covering stents of these four groups after implantation for 1, 7 and 28 days, respectively. Actb served as a control. The expression level of each individual gene on BMS group at day 28 was taken as 1. **p<0.01 vs. BMS; ++p<0.01 vs. BMS; \$\$p<0.01 vs. RAP. n=3/group.

respectively. At day 7, no stent restenosis was observed in any stent tested (Fig. 3A, upper panels). At day 28, restenosis was developed on all of these stents (Fig. 3A, lower panels); however, the restenosis was lighter on the RAP and RAP/CS/HEP compared with that on the other two group stents (Fig. 3A, lower panels). Consistent with this observation, comparison of neointimal thickness among these groups revealed that it was significantly reduced on RAP and RAP/CS/HEP compared with BMS (Fig. 3B, * p<0.05, ** p<0.01. n=3/group), while there was no significant difference in the neointimal thickness between CS/HEP and BMS. Thus, we conclude that the neointimal proliferation is repressed on RAP and RAP/CS/HEP.

Next, the lumen and neointimal area of stented region were compared among the four stent groups using histomorphometry. After day 28, the lumen area in RAP, CS/HEP and RAP/CS/HEP was all significantly larger than that in BMS (Fig. 3C, ##p<0.01, n=3/group), whereas RAP and RAP/CS/HEP produced more lumen area than CS/HEP (++p<0.01). In line with the above observations, the neointimal area of RAP and RAP/CS/HEP was the lowest, compared with that of CS/HEP (\$p<0.05, n=3/group) and BMS (**p<0.01, n=3/group). It is noteworthy that RAP/CS/HEP stents showed a significant difference in both of these two structural indices compared with CS/HEP stents. These findings demonstrate

that RAP/CS/HEP maintains the effectiveness of RAP in preventing restenosis compared with BMS and CS/HEP.

RAP/CS/HEP is efficient in promoting re-endothelialisation in porcine coronary balloon injury model

We next explored re-endothelialisation associated with the RAP/CS/HEP stent implantation in the porcine coronary balloon injury model. The implanted stents from BMS, RAP, CS/HEP and RAP/CS/HEP groups were explanted on the 7th day after implantation and fixed in glutaraldehyde, and the stent re-endothelialisation was examined using SEM. Fig. 4A shows that the endothelial cells were observed on both BMS and RAP; however, these cells were round and/or not well organized, indicating an early stage of endothelialisation. In contrast, RAP/CS/HEP and CS/HEP stents were completely covered by confluent shuttle-like endothelial cells, which formed a continuous mat and were aligned toward the blood flow direction.

The endothelialisation on these implanted four group stents was further analyzed using immunofluorescence staining against CD31, an endothelial marker, using confocal laser scanning microscope (Fig. 4B). The average coverage of endothelium on the stents of different groups was quantified and compared as described in the Methods section. Both CS/HEP and RAP/CS/HEP showed a significant improvement in endothelial coverage compared with BMS (** $p < 0.01$, $n = 6$ /group) or RAP (+ $p < 0.01$, $n = 6$ /group); however, no significant difference was observed between RAP/CS/HEP and CS/HEP in terms of endothelium coverage. To confirm this observation, RT-qPCR was carried out on the RNA purified from the tissues covering the four types of stents, using specifically-designed oligos against two endothelial functional genes, VEGF and eNOS, and two endothelial marker genes, CD31 and CD34, at days 1, 7 and 28 after stent implantation, respectively. Atcb was used as a control. Fig. 4C shows that all four types of stents exhibited no significant difference in the expression levels of these genes at day 1. At both day 7 and 28, these gene expression levels were greatly increased in both CS/HEP and RAP/CS/HEP groups compared with those in BMS (** $p < 0.01$, $n = 3$ /group), while RAP showed a significant delay in endothelial gene expression compared with BMS (+ $p < 0.01$) and with CS/HEP and RAP/CS/HEP (\$ $p < 0.01$). Thus, RAP/CS/HEP facilitated endothelial gene expression as potently as CS/HEP stents. Collectively, these data indicate that the abluminal coating of rapamycin to the CS/HEP stents improves re-endothelialisation kinetics compared with the traditional circumferential RAP stents that delay re-endothelialisation.

Discussion

The occurrence of late stent thrombosis associated with DES application has long been a clinical concern, and often requires dual antiplatelet treatment. Late stent thrombosis may be attributed to one or more potential causes, including impaired endothelial function. DES-induced delay in re-endothelialisation plays a major role in the development of late stent thrombosis. Our previous work lead to the development of a CS/HEP stent that showed both anticoagulation and endothelialisation acceleration compared with BMS [17]. Based on this prior study, we have now developed a new DES (RAP/CS/HEP) in which rapamycin is abluminally-loaded into a CS/HEP stent. The new RAP/CS/HEP exhibited high efficiency in antagonizing thrombosis *in vitro* and in large animal modes. This strong anticoagulation of RAP/CS/HEP led to an enlarged lumen area in the stented area, allowing for a smoother blood flow than seen in the BMS and CS/HEP stents. Compared with CS/HEP, abluminal coating of rapamycin also improved prevention of restenosis.

As mentioned earlier, one major disadvantage of using the traditional DES is that it delayed endothelialisation compared with BMS [20,21]. Indeed, our studies suggested that although RAP improved neointimal thickness and lumen area to the same extent as RAP/CS/HEP, it delayed reendothelialisation compared with BMS, as evidenced by the lower expression levels of endothelial markers on the tissue covering it. RAP/CS/HEP promoted re-endothelialisation to the same extent as CS/HEP as observed by the extensive coverage of endothelial cells and by the increased expression of endothelial genes. This property of RAP/CS/HEP stents is critically important because the delayed re-endothelialisation caused by the traditional version of rapamycin DES is heavily linked to late stent thrombosis and is appreciated significant concern in the clinic. Thus, compared with the old version of DES, the RAP/CS/HEP stents showed promising impacts on accelerating early phase of endothelialisation, which is an important step in vascular repair.

In summary, a preclinical study in three porcine coronary artery models showed that a newly designed DES (RAP/CS/HEP) exhibited enhanced anti-thrombosis and anti-neointimal proliferation functions but did not induce an undesired delay in re-endothelialisation. Some limitations in this study need to be pointed out; for instance, the dose-dependent effects of this RAP/CS/HEP stent need to be further characterized in both animal models and patients. In addition, the beneficial impacts of RAP/CS/HEP should be further evaluated in comparison with the new generation of DES (i.e., zota-

rolimus- and everolimus eluting stents) [22], prior to its clinical use.

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