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In vivo evaluation of cenderitide-eluting stent (CES) II

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Abstract

The use of drug-eluting coronary stents has led to significant reduction in *in-stent* restenosis (ISR), but led to delayed endothelialization, necessitating the prolonged use of expensive anti-thrombotic drugs with their side-effects. Cenderitide (CD-NP) is a novel anti-proliferative chimeric peptide of semi-endothelial origin. Our previous work *in vitro* has demonstrated; that the smooth muscle cells (SMCs) were inhibited significantly more than endothelial cells (ECs) which is the desirable feature of an anti-restenosis drug. This work reports the effects of implantation of a cenderitide-eluting stent (CES) on *in-stent* restenosis and endothelialization in an *in vivo* model. cenderitide-eluting stents were produced by coating bare metallic stents with CD-NP entrapped in biodegradable poly(ϵ -caprolactone) using an ultrasonic spray coater. A total of 32 stents were successfully implanted into 16 pigs, and all animal survived for 28 days. The plasma levels of CD-NP were significantly higher in the CES group than in the control group (bare metal stents and polymer coated stent) at post-stenting, indicating the successful release of CD-NP into blood stream *in vivo*. Furthermore, SEM analysis results showed the greater endothelial coverage of the stent struts, as well as between the struts in CES group. Moreover, histological results showed mild inflammation, and low fibrin score at 28 days. However, plasma cGMP (second messenger, cyclic 3',5' guanosine monophosphate) does not show a significant difference, and the CES is also unable to show significant difference in terms on neointimal area and stenosis, in comparison to BMS at 28 days.

Keywords

stent; natriuretic peptide; Cenderitide (CD-NP); *in vivo*

1. Introduction

Stent-based drug delivery emerged a decade ago as an effective means of reducing neointimal hyperplasia by providing localized anti-proliferative drug that suppresses the

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proliferation of smooth muscle cells in the stented segment of the artery^{14, 45}. However, the anti-proliferative agents — either cytostatic (limus-type) and cytotoxic (paclitaxel) drugs do not exhibit selectivity toward the targeted cell types; thus they not only inhibit the proliferation of vascular smooth muscle cells (VSMCs) responsible for neointimal formation, but also compromise endothelial lining repair. All the first generation DESs have significantly increased risk of late-stage stent thrombosis (LST) which is often fatal^{16, 19, 35, 44}.

Cenderitide (CD-NP), developed by the Mayo Clinic²⁴, is a hybrid of native c-type natriuretic peptide from human endothelial cells, and a C-terminus isolated from the dendroapais natriuretic peptide found in the venom of the green mamba snake. The addition of the C-terminus tail enhanced its resistance to proteolysis, resulting in increased half-life and prolonged biological action⁹. Furthermore, the C-terminus tail equips cenderitide with cardio-protective abilities to regulate natriuresis and diuresis^{9, 31, 47}. CD-NP is derived from CNP (which is of endothelial origin); hence we postulate that its semi-endothelial nature is not likely to inhibit ECs. Our previous work has demonstrated that controlled release of CD-NP from films and patches sustained the suppression of human cardiac fibroblast proliferation for cardiac remodelling indication²⁸.

Our previous work also showed that while the released cenderitide from cenderitide-eluting stent (CES) inhibits HCaSMCs proliferation it did not hamper HUVECs proliferation *in vitro*³⁰. In this work, we report on the *in vivo* performance of cenderitide eluting stent in a pig model over 28 days.

2. Materials and Methods

2.1 Materials

CD-NP (Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-Pro-Ser-Leu-Arg-Asp-Pro-Arg-Pro-Asn-Ala-Pro-Ser-Thr-Ser-Ala) was obtained from American Peptide Company, and the structure as shown in figure 1. PCL (Mn: 80,000 g/mol) (Sigma-Aldrich), PEG (Mw: 2,000 g/mol) (Merck-Schuchardt) and copolymer PCL (1.5k)-PEG (5k) (Advanced Polymer Materials Inc.) were used as receive. Phosphate buffer solution (PBS), pH 7.4 was obtained from OHME Scientific.

2.2 Coated stent preparation

The stents used in this study are closed cell design cobalt-chromium stents with strut dimensions 0.075 mm × 0.080 mm (W × T) (Fortimedix B.V., Netherlands). Bare metallic Co-Cr stents were cleaned by immersing in acetone, ethanol and distilled water for ultrasonic cleaning for 20 minutes each and dried in 60 °C vacuum oven overnight. The Sono-Tek's MedicoatTM was used to spray the coat stents used in this study. Four groups have been prepared for *in vitro* drug release study: 10% CD-NP was loaded into PCL and PCL with 10% copolymers (PEG-co-PCL: 5k-1.5k; 5k-3k and 1k-10k) blends respectively. The surface morphologies of coated stent were examined by scanning electron microscopy (SEM, JSM6360, JEOL, Tokyo, Japan) at 3kV.

2.3 *In vitro* release study from coated stents

Coated stents were prepared in triplicate, and immersed in PBS and replenished at pre-determined time-points. The amount of peptide released was detected using the micro-bicinchoninic acid (BCA) protein assay (Pierce) using the UV-Vis spectrophotometer (UV-2501, Shimadzu).

2.4 *In vivo* animal study

Animal care and preparation—Experiments were performed under the NACLAR (National Advisory Committee for Laboratory Animal Research) guidelines 2004 and investigations followed the Guide for the Care and Use of Laboratory Animals, Innoheart Pte Ltd, Singapore.

The animal model used was the sus scrofa female mixed breed swine (40–50kg). They received Aspirin (300mg/day) and Plavix (75mg/day) daily for 4 days, and were pre-medicated using Atropine Sulphate (50ug/kg, IM). The animals were then anesthetized using telazol-ketamine-xylazine (TKX) cocktail solution (0.05ml/kg, IM). Analgesics (Tramadol 5mg/kg, IM) and Antibiotics (enrofloxacin 5mg/kg, IM) were administered once before surgery.

Stent implantation—Three groups were studied: Group I- 16 CESs were implanted into both Right Coronary Artery (RCA) and Left Anterior Descending Artery (LAD) of 8 pigs; Group II- 8 BMSs were implanted into both RCA and LAD of 4 pigs; and Group III- polymer BMSs (polymer coated BMS without peptide) were implanted into both RCA and LAD of 4 pigs. A total of 32 stents (randomized) were implanted in 16 pigs, with follow up to 28 days. The sizes of stents are 2.75mm×25mm (D×L), and the expanded diameter of stents are listed in Table 2.

Under general anaesthesia (1–3% isoflurane), and with a single bolus of heparin (90 IU/kg) administered prior to stent implantation, vascular assess was established through right femoral artery and vein (which is exposed by a surgical cut-down) using a 7- French introducer sheath, Coronary arteries (RCA and LAD) were catheterized. The internal diameter of the coronary arteries was calculated after angiography with the catheter as a reference. Using this information, the stent was delivered into the coronary artery using the delivery catheter. The over-stretch ratio is 1:1 to 1: 1.3. All animal received aspirin (10mg/day) and Plavix (75mg/day) until the pre-determined end-point. Analgesic and antibiotic coverage was given for the next 6 postoperative days.

Blood collection and test—6ml blood from aorta (via femoral artery access) and right atrium (RA) (via femoral vein access), was collected before and after implantation of stent, and before sacrificing the animal at 28 days (as shown in Table 1). Then the blood was placed in EDTA tubes, and immediately centrifuged at 2,500 rpm at 4 °C for 10 minutes. The plasma was stored in polystyrene tubes at –80°C for further analysis. Radio-immuno Assay (RIA) Kit obtained from Perkin Elmer Life Science was used to evaluate plasma CD-NP and cGMP concentrations^{7, 22}. The RIA assay works on competition between ¹²⁵I-peptide and CD-NPs binding onto a limited quantity of antibodies.

Animals sacrifice—After 28 days, the animals were euthanized by an excess dose of pentobarbital sodium. The hearts were excised and then perfusion-fixed in situ by 10% buffered formalin, at less than 100mm of Hg pressure. The stented coronary arterial segments were dissected out and immersion-fixed in 10% buffered formalin. Two stented arterial segments of one animal from each group, were used for SEM analysis. The rest of the stent segment in the respective coronary arteries was used for histological analysis.

SEM analysis—Intact stented arterial segments were bi-sected longitudinally to expose the lumen surface and photographed. Both-half of the stents were rinsed in sodium phosphate buffer (pH=7.4) and were then dehydrated in a graded series of ethanol-water. After critical point drying, the tissue samples were mounted and sputter-coated with gold. The samples were visualized using a scanning electron microscopy (SEM, JSM6360, JEOL, Tokyo, Japan) at 3kV.

Histological analysis—The rest of the stent segment in the respective coronary arteries were processed and embedded in resin for histological analysis. Cross sections, 100 μ m thick, were cut at proximal, mid and distal ends. Sections were ground, polished and stained with Haematoxylin and Eosin^{15, 36, 37}. These were evaluated for histology and histomorphometric measurements: 1) injury score; 2) inflammation score; 3) strut-associated fibrin content; 4) neo-intimal growth; 5) evidence of in-stent thrombosis.

Histomorphometric measurements were carried out following Schwartz et al^{36, 37}.

2.5 Statistical analysis

All data for each stent were compared by ANOVA with post hoc analysis for multiple comparisons. Significance was established by a value of $p < 0.05$. The data are expressed as mean $\pm$ SD except as noted.

3. Results

3.1 Characterization of stents

Figures 2a–c show the SEM micrograph of coated stents from PCL formulations. The surface of the stents is very smooth and uniform, and no crack or webbing is seen between struts. Figures 2d–f show the SEM micrograph of coated stents after angioplasty balloon expansion, and no delamination or other effects were observed on the surface after expansion, confirming that the coating process was able to produce coating that was able to withstand the compressive and tensile strains imparted in the stent expansion process, without cracking.

3.2 *In vitro* release from stents

The nature of the polymer used in the coating can influence the release of the drug or the CD-NP, in this case. Figure 3a plots the cumulative peptide release from the PCL coated stent and the PCL modified with 10% addition of 3 molecular weights of copolymer of polyethylene glycol (PEG) with PCL (PEG-PCL: 5k-1.5k, 5k-3k and 1k-10k) coated stents. Figure 3b plots the comparison of initial burst and subsequent release in above groups. Using a relatively large PEG segment (5k) increased the initial burst release over pure PCL

coatings, without altering the much slower subsequent release (~12% over 30 days following initial burst). The effects are partially rationalized as being due to protein-PEG co-localization²⁵. Furthermore, the PEG-co-PCL copolymer excipient also helps by making the release less sensitive to emulsification condition²⁹. Based on the *in vitro* cell studies³⁰, the last group (10% 1k PEG-10k PCL) with 10% CD-NP loading was selected for further *in vivo* study, as it gave measurable subsequent release following an initial burst of ~27%.

3.3 Plasma levels of CD-NP and cGMP

Plasma CD-NP and cGMP concentration at pre-stenting, post-stenting and 1 month in the 3 groups are illustrated in figure 4. Plasma CD-NP concentration in CES group (n=8) was 817 ± 618 pg/ml at the RA and 830 ± 346 pg/ml at the Aorta post-stenting (figure 4a). And the CD-NP levels of CES at the other two timepoints (pre-stenting and 1 month), as well as in the other control groups (BMS n=4; polyBMS, n=4) were undetectable. cGMP is a secondary messenger molecule generated when the guanylyl cyclase (GC) receptor is stimulated by natriuretic peptides as a class. However, the plasma cGMP levels in CES groups did not show any significant difference over either pre-stenting or control groups.

3.4 *In vivo* Quantitative Coronary Analysis (QCA) results

A total 32 stents were implanted into 16 animals successfully. All animals survived through the completion of the study. Table 2 summarizes the results of the QCA performed, including the size of native artery before stent implantation, the measured size of post-expanded stent, overstretch percentage and stent/artery ratio. All overstretch percentages of arteries is not greater than 10% in all cases studied. The one month follow up results showed no significant difference in the late loss.

3.5 Endothelialization at 28 days

At 28 days, a total of 6 stents (2 from each group) were analysed. The stent surface endothelialisation evaluated by SEM showed as Figure 5. All groups showed greater endothelial coverage above struts, as well as between the struts. However, there are two uncovered struts were observed in both BMS and CES, as shown in the higher magnification image in the left of Figure 5, that probably due to small vessel branch.

3.6 Histological analysis

Figure 6 summarize the histomorphometry data and Figure 7 shows the representative histological image from each group at 28 days. Both injury scores and inflammation score from 3 groups were close to 0 at 28 days, so results are not shown here. However, there are no significant difference in stenosis percentage, neointimal area, neointimal thickness and the fibrin score in 3 groups at 28 days.

4. Discussion

Re-endothelialization after stenting is a critical step in the process of vascular healing. However, in current drug-eluting stent implantation, the vascular healing is altered as there is non-selective inhibition of all cell types (SMCs and ECs), resulting in incomplete or

delayed endothelialisation of the vessel^{6, 35, 41}. The lack of adequate healing increases the chance of late-stent thrombosis with potentially fatal outcome.

In our previous *in vitro* study, we have developed a novel CES, incorporating the designer peptide, CD-NP. The coated stent is able to release up to 50% of the CD-NP at 28 days, which is required to effectively counter rapid proliferation of SMCs post-stenting^{16, 35, 44}. Moreover, our *in vitro* SMCs and ECs cell study showed that the CD-NP released from all formulations elicited elevation of cGMP, implying that bioactivity was retained. Furthermore, the cell study demonstrated that CD-NP inhibited Human coronary smooth muscle cells (HCaSMCs) but did not affect the proliferation of human umbilical vein endothelial cells (HUVECs).

In current work, a total of 32 stents were implanted into 16 animals successfully (CES=16, BMS=8 and Poly BMS=8), all animals survived until 28days, the plasma CD-NP, plasma cGMP, injury score, inflammation score, endothelialization, neointimal area, neointimal thickness and percentage of stenosis were evaluated.

A brisk early inflammatory response was produced after balloon injury or stent placement with abundant surface-adherent leukocytes of monocyte and granulocyte lineage^{33, 46}. Days and weeks later, macrophages invaded the forming neointimal tissue and were observed clustering around the struts, forming giant cells. In this study, the mean inflammation is mild in all groups. Furthermore, the cobalt chromium stent has higher tensile modulus, greater yield strength, greater elongation-to-break and greater density, in comparison with stainless steel stent. Therefore, the Co-Cr stent is able to maintain the same mechanical stability with much thinner stent struts, which results in greater stent flexibility in curved lesions, and a more filigree design²⁰. Results of a porcine model over 4 weeks clearly showed that using the CoCr stent as a base for DES instead of the 316L stainless steel, the achieved anti-inflammatory effect of the drug-loaded stent is consistently more^{3, 12, 13, 17, 43}. For this study, the mild intimal inflammation responses observed in both CES and BMS groups, an even in the pure polymer coated stent group, were due to the use of the Co-Cr stent.

In our previous work¹⁶, we have reported that the % stenosis of PLGA coated Co-Cr stent is significantly higher than the Co-Cr BMS in a porcine model over 4 weeks (BMS=30±4%, and PLGA-coated stent = 40±10%, p=0.011). In the current study, the PCL-coated stent shows a comparable stenosis percentage with the Co-Cr bare stent. PCL, poly-lactic acid (PLA) and poly-lactic-co-glycolic (PLGA) have been widely used in drug delivery, sutures and medical devices due to good biocompatibility and degradability¹⁴.

Endothelium is an important vascular component that regulates the synthesis of growth factors, nitric oxide and protein matrixes³⁵, and its dysfunction promotes platelet adhesion and subsequent thrombosis. In the last 10 years, there have been escalating research effort on the use of natriuretic peptide (NP) as therapeutic agents^{1, 31, 32, 47} in place of synthetic drugs. In particular, the c-type natriuretic peptide (CNP) is a potent vasodilator and anti-inflammatory agent^{2, 5, 18, 38}. In a rabbits study³⁹, continuous infusion of CNP post balloon catheter injury resulted in suppressed neointima formation. However, the short half-life of CNP and lack of cardio-renal functions limits its utility in disease states requiring chronic

administration, such as coronary artery disease. CD-NP, developed by the Mayo Clinic²³, is a hybrid of native c-type natriuretic peptide from human and a C-terminus isolated from the dendroaspis natriuretic peptide found in the venom of the green mamba. The addition of the C-terminus tail enhanced the resistance to proteolysis, resulting in increased half-life and prolonged biological actions⁸. Furthermore, the C-terminus tail equips CD-NP with cardio-protective abilities to regulate natriuresis and diuresis^{8, 27, 48}. CD-NP originates from endothelial origin CNP, hence we believe it is partial endothelial in nature. We postulate that its semi-endothelial nature is not likely to inhibit ECs. Our *in vitro* cell studies³⁰ showed that CD-NP did not inhibit the growth of ECs, suggesting that CD-NP displayed cell-type discrimination inhibition. Our current *in vivo* study showed that CES has almost 100% endothelial coverage above struts, and between struts at 28 days.

However, the CES group is not able to show significantly different outcome in terms on neointimal area and neointimal thickness and stenosis, in comparison to BMS. The native CNP has been reported to mediate the inhibition of SMCs but promotes the proliferation of HUVECs^{10, 11, 40, 47}. In SMCs, CNP mediates inhibition via NPR-C activation (cGMP production) and ERK 1/2 phosphorylation. ERK 1/2 in SMCs triggers the expression of p21waf1/cip1 and p27kip1 expression, thereby preventing cell cycle progression and proliferation. On the other hand, CNP induces cell proliferation in HUVEC via NPR-C activation and increase in ERK 1/2 and Akt activation. The activation results in augmenting cyclin D1 expression, which facilitates cell cycle progression. Therefore, CNP-induced cGMP regulates cell cycle protein expression and hence proliferation differently in SMCs and HUVEC. However, in current study, although the level of plasma CD-NP post-stenting was significantly higher for the CES group than pre-stenting and the control groups, the plasma cGMP concentration in CES groups was not significantly different. Our previous *in vitro* study showed that the CD-NP elicited cGMP production in a dose-dependent manner similar to native NPs^{31, 47}. On the other hand, oversizing the coronary artery results in a degree of vascular injury after stenting, that leads to variable amounts of inflammation, neointimal proliferation and restenosis. In this study, the stent to artery ratio is slightly higher as 1.10 ± 0.09 , that could be contributor to the overall higher percentage of restenosis rate in all three groups.

We believe that the dosing of CD-NP (2 micrograms/day over 28 days) used in this study was not sufficient to activate sufficient cGMP locally or systemically to have a significant effect on SMC proliferation. On the other hand, the CES did exhibit the greater endothelial coverage of the stent struts, as well as between the struts, perhaps indicating a beneficial effect on endothelialisation even at this low dosage. Apart from inhibiting SMCs proliferation, ensuring unperturbed endothelium regeneration is crucial to avert further progression of ISR and LST.

5. Conclusion

We have successfully developed a Cenderitide-eluting stents (CES) by coating bare metallic stents with CD-NP entrapped in poly(ϵ -caprolactone) using an ultrasonic spray coater. A total of 32 stents were successfully implanted into 16 pigs, and all animal survived for 28 days. The plasma levels of CD-NP were significantly higher in the CES group than in the

control group at post-stenting, indicating the successful release of CD-NP into blood stream *in vivo*. Furthermore, SEM analysis results showed the greater endothelial coverage of the stent struts, as well as between the struts in CES group. Moreover, histological results showed mild inflammation, and low fibrin score at 28 days. However, plasma cGMP does not show a significant difference, and the CES is also unable to show significant difference in terms on neointimal area and stenosis, in comparison to BMS at 28 days.

Limitation

It has been reported immobilization of anti-CD34 antibody on sirolimus eluting stent enhances endothelialization at 3 days (SES-anti-CD34 $36\pm 26\%$, SES $7\pm 3\%$), and 14 days (SES-anti-CD34 $82\pm 8\%$, SES $53\pm 20\%$)²⁶. However, in current study, the examination time point was predetermined at 28 days, and all groups show almost completely endothelialisation at 28 days, it may possible to pick up differences at early time, such as 3 days or 14 days.

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Figure 1.

Amino acid (AA) sequence and structure of a CNP and chimeric natriuretic peptide C-terminus of DNP and CD-NP.

(c-type natriuretic peptide (CNP) is a 22-amino acid (AA) endothelial-cell-derived natriuretic peptide, C-terminus of dendroaspis natriuretic peptide (DNP) is a 15-AA chimeric natriuretic peptide, and CD-NP is a 37-AA-designed chimeric natriuretic peptide)

Figure 2.

SEM images of the CESs: (a) the overview of the pre-expansion CES; (b) the outer surface of the pre-expansion CES; (c) the side surface of the pre-expansion CES; (d) the overview of the post-expansion CES; (e) the outer surface of the post-expansion CES; and the side surface of the post-expansion CES.

Figure 3.

In vitro CDNP release profiles from CESs: (a) the cumulative peptide release from the PCL coated stent and the PCL modified with 10% addition of 3 molecular weights of copolymer of polyethylene glycol (PEG) with PCL (PEG-PCL: 5k-1.5k, 5k-3k and 1k-10k) coated stents (n=3); and (b) the comparison of initial burst and subsequent release in above groups (n=3).

Figure 4.

In vivo plasma CD-NP and the plasma cyclic 3'/5' guanosine monophosphate (cGMP): (a) The comparison of the plasma CD-NP in CES group at pre-stenting, post-stenting and 1month (CES n=8); and (b) the comparison of the plasma cGMP in BMS, CES and PolyBMS at pre-stenting, post-stenting and 1 month. (CES n=8, BMS n=4, and polyBMS n=4).

Figure 5. Representative SEM images of lumen surface in CES, BMS and PolyBMS at 28 days (n=2). (Arrows point to the area with higher magnification is shown on the left)

Figure 6.

The comparison of stenosis percentage (A), neointimal area (B), neointimal thickness (C), and fibrin score (D) in CESs, BMSs and PolyBMSs at 28 days (CES n=21, BMS n=9, and PolyBMS n=9, $p > 0.05$).

Figure 7. Representative histological images of overview of stented artery (Right Coronary Artery (RCA) and Left Anterior Descending Artery (LAD)) at 28 days showing the comparison in neointimal area in CESs, BMSs and PolyBMSs.

Table 1

Study timeline: Experimental protocol of the stent in the animal model.

Table 2*In vivo* quantitative coronary analysis (QCA) results

Sample Name	Native Artery (mm)	Expanded Stent (mm)	Overstretch (%)	Stent/Artery Ratio	late lumen loss (mm)	n
CES	2.60±0.36	2.83±0.32	8.85±0.34	1.1±0.08	1.17±0.42	16
BMS	2.79±0.22	3.00±0.28	7.53±0.25	1.09±0.11	0.76±0.50	8
Poly BMS	2.49±0.19	2.74±0.16	10.04±0.18	1.1±0.08	0.89±0.46	8