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Use of a novel anti-proliferative compound coated on a biopolymer to mitigate platelet-derived growth factor-induced proliferation in human aortic smooth muscle cells: comparison with sirolimus

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Abstract Drug eluting stents (DES) have become a common mode of treatment for stenosis in coronary arteries. However, currently, the use of sirolimus/paclitaxel-coated DES has come under scrutiny, because of their prothrombotic effects leading to potential adverse outcomes in the long run. We have previously documented that D-threo-1-phenyl-2-decanoylamino-3-morholino propanol (D-PDMP); an inhibitor of glucosylceramide synthase and lactosylceramide (LacCer) synthase markedly inhibited platelet-derived growth factor (PDGF)-induced cell proliferation. We have fabricated DES wherein, D-PDMP or sirolimus was coated on to a double layer of poly (lactic-co-glycolic acid) on a bare metal stent. The *in vitro* release of D-PDMP from biopolymer and its consequent effect on PDGF induced proliferation and apoptosis was assessed in human aortic smooth muscle cells (ASMC). D-PDMP was released from biopolymers in a dose-dependent fashion and was accompanied with a decrease in PDGF-induced cell proliferation, but not apoptosis. In contrast, sirolimus markedly increased apoptosis in these cells in addition to inhibiting proliferation. Our mechanistic studies revealed that D-PDMP, but not sirolimus decreased the cellular level of glucosyl and lactosylceramide that accompanied inhibition of PDGF-induced cell proliferation. Our short-term (14 days) *in vivo* studies in rabbits also attested to the safety and biocompatibility of the D-PDMP coated stents. Our data reveal the superiority of D-PDMP coated biopolymers over sirolimus coated biopolymers in mitigating ASMC proliferation. Such D-PDMP coated stents may be useful for localized delivery of drug to mitigate neo-vascular hyperplasia and other proliferative disorders.

Keywords Lactosylceramide · Platelet derived growth factor · Aortic smooth muscle cells · Biopolymer · Drug eluting stent

Abbreviations

DPDMP	D-threo-1-phenyl-2-decanoylamino-3-morholino propanol
PDGF	platelet-derived growth factor
LacCer	lactosylceramide
GLcCer	glucosylceramide
HPLC	high performance liquid chromatography
PLGA	polylactic-co-glycolic acid
TNF	tissue necrosis factor
ASMC	aortic smooth muscle cells
DES	drug eluting stents

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Introduction

Coronary artery disease (CAD) is the leading cause of mortality and morbidity in the United States contributing 20% to all deaths [1]. The use of stents for treating CAD has been regarded as a major step forward in the field of percutaneous coronary interventions (PCI) [2]. Restenosis occurring from neo-intimal hyperplasia in long-term follow up after PCI have been a major limitation in their success [3–5]. Use of drug eluting stents has considerably reduced the restenosis rates in patients undergoing PCI [3, 4]. Currently, only two drug eluting stents (DES) are approved by the FDA for clinical usage, sirolimus eluting stents (Cypher) and paclitaxel eluting stents (Taxus). Recently, the use of these DES has been under scrutiny with reports of increased in-stent thrombosis, myocardial infarction and other major adverse cardiovascular events requiring re-intervention [6–11]. Such debates on the safety, efficacy and cost effectiveness of DES [8, 12–15] as compared to bare metal stents mitigates some of the earlier optimism and enthusiasm regarding DES use and calls for a cautionary approach to the issue of late stage thrombosis and the need to look for more efficient and novel DES.

The act of introducing a stent in a vessel results in injury to the vascular wall causing endothelial denudation and inflammation subsequently leading to a regenerative response in the vascular smooth muscle cells involving their proliferation, migration and accumulation of extracellular matrix in response to growth factors like PDGF and inflammatory cytokines like tumor necrosis factor (TNF-α) [16–19]. A DES has three main components: metal stent backbone, a non-biodegradable polymer coating and the drug contained in the coating. DES is designed on the idea of reducing restenosis by inhibiting the proliferative effects of growth factors like PDGF. This is achieved by coating conventional stents with a polymer coating loaded with an immunosuppressant or an antiproliferative drug which ensures their delivery in an effective dose to the site of injury and inflammation, thus reducing neointimal hyperplasia without reaching toxic levels in the blood [16, 20]. The biochemical nature of the polymer and its interaction with the living tissues inside the blood vessel is vital for limiting the amount of local inflammation [16]. The commonly used DES has a non-biodegradable coating that remains in the body after the drug elutes completely [16]. This may lead to complications such as exaggerated inflammatory response, local toxicity, polymer breakdown and erosion, and incompatibility with humoral factors [16, 21, 22]. It is therefore desirable to use biocompatible, biodegradable inert, non-thrombogenic polymer coatings on the stents that can tolerate mechanical stress [23, 24]. The flexibility of the stent coating is important for the film integrity during stent crimping and implantation using balloon catheters as cracking or peeling of the coating may cause severe thrombosis and inflammation [25]. DES met with great successes initially in reducing restenosis rates though there were reports of delayed healing in autopsy and animal studies [26, 27].

CAD is an inflammatory and proliferative disorder [28, 29]. Herein, several growth factors, cytokines and oxidized-low density lipoproteins (Ox-LDL) play critical roles that contribute to endothelial dysfunction such as the expression of cell adhesion molecules [30, 31], angiogenesis [32] and smooth muscle cell migration and proliferation [33–38]. Most remarkably, we observed that Ox-LDL, PDGF, VEGF and TNF induced phenotypic change in vascular cells required the activation of an enzyme lactosylceramide (LacCer) synthase which converts a glycosphingolipid, glucosylceramide to LacCer. In turn, LacCer activated an "oxygen sensitive" signaling pathway that led to the phenotypic changes described above [39, 40]. Interestingly, the use of D-PDMP; an inhibitor of GlcCer synthase and LacCer synthase mitigated such phenotypic changes. Therefore, we rationalized that D-PDMP may well be a suitable candidate in DES to mitigate vascular smooth muscle cell proliferation induced by PDGF.

Here we describe our approach to use D-PDMP in a coating on a metal stent. Specifically, D-PDMP was loaded on a double layer of polylactic-co-glycolic acid (PLGA) a biodegradable polymer coating on a bare metal stent. PLGA has been accepted in many applications that involve implantation or injection into the body like controlled delivery of protein and peptide drugs, manufacture of sutures and wound dressings as well as for fabricating scaffolds in tissue engineering [41–44]. PLGA and related compounds have been used previously as a polymer coating on stents for *in vitro* studies [45, 46]. In our experiment to counter delamination or film detachment, a double layer was designed. The first layer, made of amorphous PLGA, serves mainly as an adhesion promoter between the top layer and the stainless steel substrate. And the top layer, semi crystalline PLGA, is the D-PDMP matrix and provides required mechanical strength and control of drug release for desired duration. This design is considered to be of better reliability and integrity as compared to the coating only on the outer surface of the struts during crimping and expansion. For comparison purpose, we have also used sirolimus coated DES in our studies.

Our data reveal that D-PDMP coated on biodegradable matrix material is efficiently released and can dose-dependently mitigate PDGF induced human ASMC proliferation without inducing apoptotic death. Moreover, D-PDMP coated stents were resistant to crimping and stretching in our *in vivo* experiments in rabbits.

Materials and methods

Materials

Two types of polylactic-co-glycolic acid (PLGA, both from Purac, Netherlands) were selected in this study, including PLGA 80/20 (IV 1.7–2.6) and PLGA 53/47 (IV 0.97). Dichloromethane (HPLC grade, Aldrich), chloroform (HPLC grade, Aldrich), ethyl acetate (ACS reagent, Aldrich) were standard laboratory agents and used as received. Bare metal stents (BMS) in a dimension of 14×3.5 mm (length X OD when fully expanded) were purchased from Luminous Co, USA. D-PDMP was purchased from Matreya Biochemicals (Pleasant Gap, Pennsylvania, USA). [^{14}C] Palmitic acid (specific activity 50–60 mCi/mmol) and [^{3}H] Thymidine (20–30 Ci/mmol) were purchased from American Radio labeled Chemicals Inc. (St Louis, Missouri). BCA protein assay kit was obtained form Pierce (Rockford, Illinois). Human ASMC and culture medium were purchased from Cascade Biologics (Portland, Oregon). PDGF-BB was purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

The materials used in the animal trial include the following: Pentobarbital sodium was from Shanghai, China. Heparin sodium solution was purchased from Shanghai 1st Biochemical and Pharmaceutical Corp., China. Aspirin (tablet) was from Kunming Pharmaceutical Corp., China. Warfarin sodium (3-(α-Acetonylbenzyl)-4-hydroxycoumarin sodium salt) was purchased from Shanghai Pharmaceutical (Group) Co. (China). Ceftriaxone sodium was from Kunming Jida Pharmaceutical Co. (China). Glucose and dextrose were from Kunming Nanjiang Pharmaceutical Co., Kunming (China). Glofusine was purchased from Shenyang B. Braun Pharmaceutical Co. Ltd., Shenyang (China).

Methods

1. *Stent coating with PLGA and loading of DPDMP on the polymer*: Stent preparation for the experiments conducted was done as described below:
   (a) *Spraying fixture and enclosure*: The spray coating process was carried out in an enclosure. A bare metal stent was fixed on a stent holder that was connected to the rotor. Hence, the stent could rotate and move horizontally at a designated speed. The airbrush (Badger, USA) could be placed at an angle of 0° to 90° and at a distance of 0 to 15 cm.
   (b) *Pre-spray materials preparation*: The prescribed amount of PLGA was dissolved in either ethyl acetate (for 53/47 IV 0.97) or a mixture of ethyl acetate and chloroform (50/50, for 80/20 IV 1.7–2.6). The drug-free solutions were stored in sealed glass bottles at room temperature. D-PDMP was dissolved and sonicated in aliquot volume of chloroform for 5 min. The drug solution and PLGA in ethyl acetate or ethyl acetate and chloroform solution were mixed in appropriate proportions to achieve a desired concentration. These solutions were stored in sealed opaque glass bottles at −20°C.
   (c) *Metal stent preparation*: Fresh metal stents were soaked and sonicated in acetone, ethanol and pure water consecutively, each for 1 h. The clean stents were recovered and oven dried at 55°C and stored in desiccators.
   (d) *Airbrush or spray coating*: A metal stent was fixed onto the stent holder shown in Fig. 1 and was

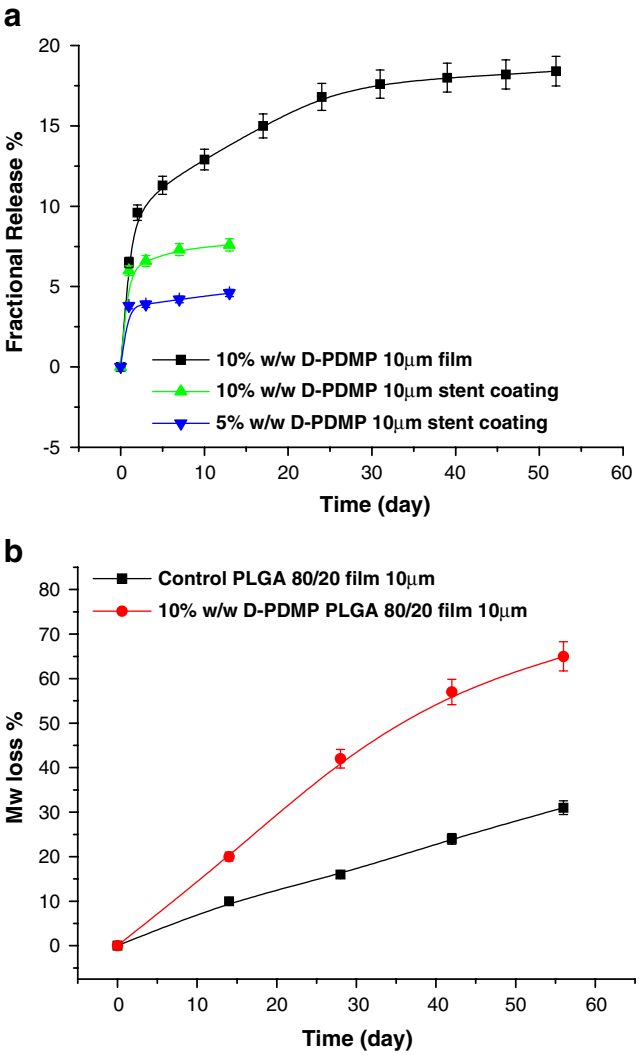

Fig. 1 **a** D-PDMP release from the PLGA film and coated stents. **b** Molecular weight loss percentage of PLGA 80/20 films

rotated at 25 rpm. The stent also moves horizontally and reciprocally at an average velocity of 60–80 mm/s. The airbrush was fixed at the same vertical height as the stent, at a 100 mm distant from the rotating axis, with its nozzle perpendicular to the axis and in line with the free end of the stent. The amorphous PLGA solution was sprayed at about 250 μL/min onto the metal stent to produce the first coating layer. The semi-crystalline PLGA solution was sprayed at the same rate after the primer layer was air dried for 1 h. PDMP-containing solutions were sprayed under subdued light, as the drug is sensitive to light, heat and humidity. The sprayed stents were collected, kept in opaque vials and vacuum dried at room temperature for 24 h. D-PDMP-free stents were stored in desiccators at room temperature while D-PDMP containing stents were stored in freezers at −20°C.

(e) *Stent sterilization and packaging for animal trial*: Coated stents were first restored to room temperature in desiccators if they were previously stored in freezers. The stents were then crimped onto balloons of 16×3.0 mm (length × OD, designed for 14 mm-length stent) at one end of the coronary catheters at room temperature on a pneumatic crimper (SC100, Machine Solutions Inc. U.S.A.) at Merlin MD Pvt. Ltd., Singapore. The stented catheters were sealed in ethylene oxide (EO)-permeable pouches and EO sterilized at 37°C for 6 h at Tan Tock Seng Hospital, Singapore. Post-sterilization cell culture study verified the efficacy of EO sterilization. These sterilized stents were used in the 14-day rabbit model trial.

2. *In vitro* degradation and drug release

(a) *Preparation of PLGA films and coated stents*: PLGA films were prepared by casting PLGA dichloromethane solutions of prescribed concentrations on glass plates. Wet films were dried at room temperature for 4 h and subsequently dried in an oven at 40°C for 3 days. For drug loaded films, D-PDMP was suspended in a small aliquot of dichloromethane and sonicated for 3 min. Prescribed amount of PLGA dichloromethane solution was added into the drug suspension and was mixed well with a pipette. The mixture was sonicated for 30 s to remove remaining bubbles. The D-PDMP containing PLGA solution was cast into films on glass plates, air dried and then oven dried at the same experimental conditions as the above. 10 μm thick PLGA films with 10% *w*/*w* D-PDMP were prepared for *in vitro* drug release study. Coated stents of 5% *w*/*w* and 10% *w*/*w* D-PDMP loadings were prepared through the air brush procedure described earlier and sampling were done at day 1, 3, 7 and 13 for high performance liquid chromatography (HPLC) measurement.

(b) *Drug quantification*: Either PLGA films or coated stents were submerged in phosphate buffer solution (PBS). Films were immersed in PBS for 52 days, while coated stents were immersed in PBS for 14 days. Samples were filtered and injected into HPLC 1100 (Agilent Technologies, USA) at designated time points. The quantification of drug concentrations was done immediately after sampling.

(c) *Degradation study*: Degradation study was carried out in both control and 5 and 10 μm thick D-PDMP loaded films. Films were cut to 20×20 mm and immersed in PBS. Samples were removed and dried at regular time intervals. Gel permeation chromatography (GPC 1100, Agilent Technologies USA) was performed to measure the change in molecular weight.

3. *In vitro* cell culture experiments

(a) *Apoptosis assay*: 1×10^3 human ASMC were grown on sterilized glass cover slips in 6x well trays and treated with media and control wafer alone (control), control wafer and PDGF (25 ng/mL), 1% D-PDMP wafer (18.98 μg/cm^2, 10 wafers/well), 5% D-PDMP wafer (92.12 ng/cm^2, 10 wafers/well), 1% Sirolimus wafer (18.98 μg/cm^2, 10 wafers/well), with C_2-ceramide (10 μmol/L, positive control), D-PDMP (10 μmol/L) alone, D-PDMP (10 μmol/L) and 2% fetal bovine serum, sirolimus (10 μmol/L) and 2% fetal bovine serum, Sirolimus alone (10 μmol/L) and 2% fetal bovine serum alone for 24 h. After 24 h incubation, the medium was removed, cells fixed with ethanol: acetic acid 3:1 (*v*/*v*),washed three times with phosphate buffer saline and stained with DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride) reagent and nuclei were visualized by fluorescence microscopy (Zeiss Axiovert 25).

(b) *Proliferation assay*: Semi confluent cultures (~80% confluent) of human ASMC were grown in 96-well sterile plastic plates with 1×10^4 cells per well. Next, they were incubated with 100 μL medium along with control wafers alone, 25 ng/mL PDGF alone, PDGF (25 ng/mL) and 1% sirolimus wafer (1 wafer/well, 18.98 μg/cm^2), PDGF and sirolimus (10 μmol/L), PDGF and 1%D-PDMP (1 wafer/well, 18.98 μg/cm^2), PDGF and 5% D-PDMP (92.12 μg/cm^2, 1 wafer/well), PDGF+D-PDMP (10 μmol/L) in two sets of six replicates

each for 18 and 36 h, respectively. Next at 18 and 36 h [^{3}H] Thymidine (5 μCi/mL) was added and incubation was continued for another 6 h. The reaction was terminated by removing the wafers/medium and washing with PBS. Then the cells were dissolved in 100 μL of 1 N NaOH and left for drying overnight. Later the cells were re-suspended in 100 μL of water and dissolved in Econosafe, scintillation fluid, and radio scintillation counting was done in a Beckman liquid scintillation counting system.

(c) *Measurement of glycosphingolipids*: Confluent cell cultures of human ASMC grown in six-well plates were incubated with [^{14}C] palmitate (2 μCi/mL) then these cells were incubated in two sets of triplicates for 24 and 48 h with PDGF (25 ng/mL) alone, control wafer, PDGF+sirolimus (10 μmol/L), PDGF+sirolimus (5 μmol/L), PDGF+D-PDMP (10 μmol/L), PDGF+DPDMP (5 μmol/L), PDGF+1%D-PDMP wafers (18.98 μg/cm^2 of D-PDMP on wafers,10 wafers /well), PDGF+5%D-PDMP wafers (total of 92.12 μg/cm^2 of D-PDMP on wafers, 10 wafers/well), PDGF+1% sirolimus wafers (18.98 μg/cm^2 of sirolimus on wafers, 10 wafers/well), PDGF+5% sirolimus 92.12 μg/cm^2 of sirolimus on wafers, 10 wafers/well). After incubation for 24 and 48 h, cells were washed three times with PBS and lipids were extracted with hexane–isopropanol (3:2, by volume) for 15 min at room temperature. The procedure was repeated and the lipid extracts were pooled and dried in nitrogen. The total lipid extracts were solubilized in chloroform and subjected to fractionation by silicic acid column chromatography (PREPSEP extraction columns, Fisher Scientific).Then the glycosphingolipid fractions (acetone–methanol wash) were separated by high performance thin layer chromatography along with standard glycosphingolipids using chloroform–methanol–water (100:42:6 *v*/*v*) as solvent. Following autoradiography the lactosylceramide and glucosylceramide gel areas were excised and radioactivity was measured using liquid scintillation counting fluid and Beckman scintillation counter. The solvent extracted cell culture dish was solubilized over night with 1 mol/L NaOH and then suitable aliquots was withdrawn for the measurement of protein using the BCA protein assay kit.

4. *In vivo* stent evaluation in a rabbit model

Four types of stents were evaluated in this rabbit model for 14 days (Table 1). A total number of 12 rabbits (body weight 3–4 kg) were studied in this trial. Aspirin (50 mg/day) and warfarin sodium (0.5 mg/day) were fed to rabbits orally 3 days before surgery. Aspirin (50 mg/day) was continued for another 2 days after that. About 10 mL of blood was drawn for each rabbit 2 days before stenting. Rabbits were anesthetized by 3% pentobarbital sodium (IV) at a dose of 1 mL/kg body weight and were fixed to the operating worktable. The hair on surgery site was removed and sterilized with iodine and 70% ethanol. The right femoral artery was separated and exposed. The distal end of the separated femoral artery was ligated and a small cut was made at the proximal end. Heparin was given at a dose of 600 IU/rabbit. A 5F artery sheath was inserted from the cut and the stent mounted on balloon was delivered into right femoral artery by catheter. The balloon was inflated and the stent was fully expanded. Ultravist® (Iopromide) 769 mg/mL/10 mL per angiogram session/rabbit was injected into artery and the X-ray image of the transient radio contrast distribution was recorded by digital subtraction angiography (DSA). The proximal end of the artery was ligated after stenting and the wound was stitched. Ceftriaxone sodium (0.5 g/rabbit) was injected (SC) at the site of the wound. The 30 mL 5% glucose and 20 mL dextrose solution were given (IV) after surgery. The above animal protocol was approved by the institutional committee on research on animals at the Nan-Yang Technical University, Singapore.

Results

Drug release profiles and degradation

There is a burst release of about 4% to 9% of the total loading for all samples at day 1, (Fig. 1a), mostly because

Table 1 Stent preparation for rabbit model trial

Description	Coating	D-PDMP (%)
Control 1	Bare metal stent	–
Control 2	PLGA 53/47 and 80/20 double layer	–
Sample 1	PLGA 53/47 and 80/20 double layer	5
Sample 2	PLGA 53/47 and 80/20 double layer	10

there are some hydrophilic D-PDMP particles exposed on the film surface. From day 2 onwards, all the profiles show a typical first-order diffusion-controlled release of D-PDMP. Based on these *in vitro* release results, D-PDMP release is sustainable for more than 8 weeks. The lower release obtained from stent coatings may be attributed to the uni-directionality of the release from the coated stents.

Comparing the molecular weight loss percentage of 10% *w/w* D-PDMP films and control films (Fig. 1b), it is noted that at week 8, the D-PDMP loaded films lost about 60% of the original MW while control films only lost less than 40%. Hence D-PDMP contributes to faster degradation of the PLGA 80/20 films. This is not surprising considering the slightly basic nature of PDMP; we have observed similar autocatalytic effects with basic and acidic drugs [8]. Based on the drug release profiles and *in vitro* degradation results, it can be concluded that the PLGA coated stents are able to maintain an 8-week D-PDMP release.

In vitro cell culture effects of the DPDMP coated polymers

D-PDMP coated biopolymer wafer exerts a sustained inhibition of cell proliferation

PDGF markedly stimulated the proliferation of human ASMC in 24 h. And this was mitigated by sirolimus alone and 1% sirolimus coated biopolymer wafers (Fig. 2). Incubation of cells with 1% D-PDMP and 5% D-PDMP coated biopolymer wafers also markedly inhibited cell proliferation and this was modestly greater than sirolimus.

However, when cells were incubated with D-PDMP alone for 24 h we observed a twofold increase in the inhibition of cell proliferation as compared to sirolimus alone. When the time of incubation was raised to 48 h sirolimus alone and 1% sirolimus or D-PDMP coated biopolymers did not inhibit cell proliferation significantly. But D-PDMP alone and 5% D-PDMP coated biopolymer wafers continued to significantly inhibit cell proliferation. In sum, D-PDMP coated biopolymer wafers are highly efficacious in delivering D-PDMP to cells that can dose- and time-dependently inhibit the proliferation of human ASMC.

D-PDMP coated biopolymers do not induce apoptosis in cultured human ASMC in presence or absence of serum

As shown in the Fig. 3, D-PDMP did not induce any significant apoptosis in the cultured human ASMC. In serum-free medium the percentage of apoptotic cells seen following incubation with 1% DPDMP (1.09%) and 5% DPDMP (2.3%) were not much different from that seen in the control plates and was significantly lower than that seen

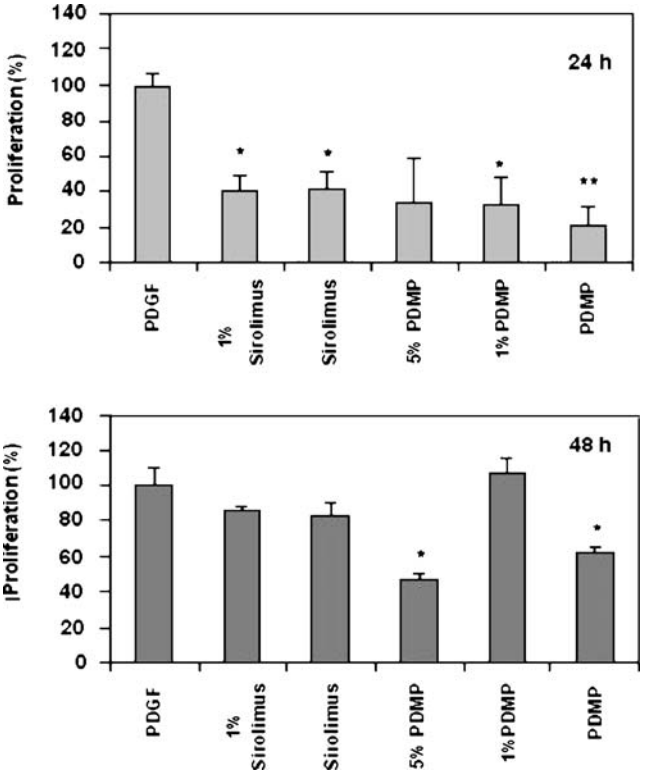

Fig. 2 Effect of D-PDMP and sirolimus on human ASMC proliferation. Semi-confluent cultures of human aortic smooth muscle cells were grown in 96-well plates and incubated with the following: control wafers alone, 25 ng/mL PDGF alone, PDGF (25 ng/mL) and 1% sirolimus wafer (1 wafer/well, 18.98 μg/cm^2), PDGF and sirolimus (10 μmol/L), PDGF and 1%D-PDMP (1 wafer/well, 18.98 μg/cm^2), PDGF and 5% D-PDMP (92.12 μg/cm^2, 1 wafer/well), PDGF+D-PDMP (10 μmol/L) in two sets of six replicates each for 18 and 36 h, respectively. After incubation for 18 and 36 h, respectively, [^{3}H] thymidine (5 μCi/mL) was added and incubation was continued for 24 and 48 h at 37°C. Next, the cells were washed and the incorporation of [^{3}H] thymidine was measured by scintillation spectrophotometry. The data is presented as percent control referring to cells incubated with PDGF alone±SD. *$p \leq 0.05$; **$p \leq 0.01$. Cells incubated with PDGF alone incorporated 2,173.37±156.71 cpm of 3H thymidine in 24 h and 417.78±41.49 cpm of 3H thymidine in 48 h and these values were used as 100% in our calculations

in sirolimus incubated cell cultures (*$p \leq 0.05$). The number of apoptotic cells was also higher following incubation with C_2 ceramide.

Similarly, in the presence of serum the percentage of apoptotic cells seen in culture incubated with sirolimus and 1% sirolimus were significantly higher (*$p \leq 0.05$, **$p \leq 0.01$ respectively); whereas, cells incubated with D-PDMP showed significantly lower levels of apoptosis (*$p \leq 0.05$). In sum, D-PDMP coated polymers do not cause any increase in apoptosis in the human ASMC and thus its inhibitory effect on cell proliferation is not mediated by a cytotoxic mechanism. However, sirolimus does increase apoptotic death of smooth muscle cells.

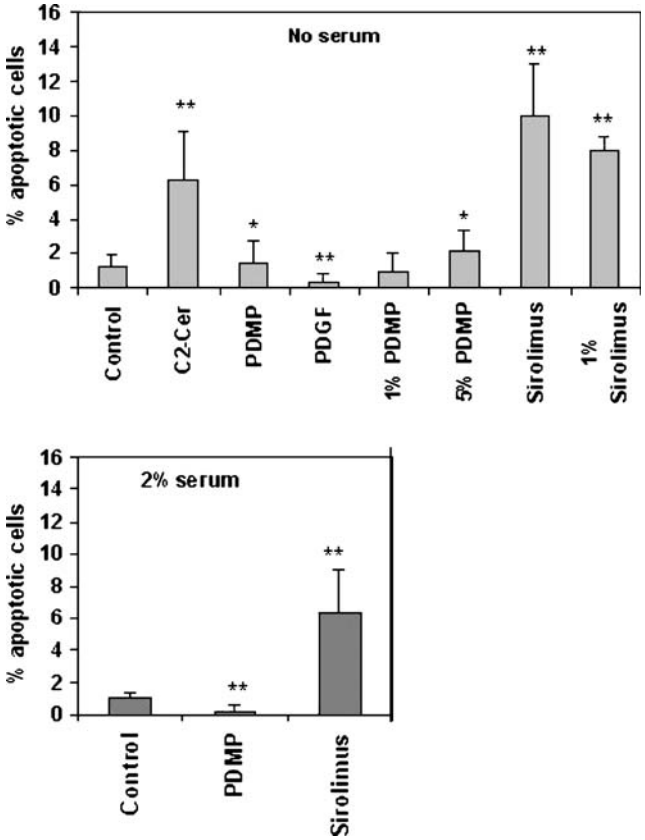

Fig. 3 Effect of sirolimus and D-PDMP on apoptosis in human ASMC. Human ASMC were grown on sterilized glass cover slips and incubated for 24 h with media and control wafer alone (control), control wafer and PDGF, 1% PDMP wafer, 5% D-PDMP wafer, 1% sirolimus wafer, with C_2-ceramide (positive control), PDMP (10 μmol/L) alone, sirolimus (10 μmol/L) alone, PDMP (10 μmol/L)+ 2% FBS (fetal bovine serum), sirolimus (10 μmol/L)+2% FBS and 2% EBS alone for 24 h. Cells were then fixed with ethanol acetic acid (3:1 volume/volume) and washed three times with phosphate-buffered saline. The cells were then stained with DAPI reagent, mounted on a glass slide and subjected to fluorescent microscopy. The cells were counted in three fields with nearly 100 cells per field and the overall percent of apoptotic cells was plotted. The values are percent values±SD.*$p \leq 0.05$; **$p \leq 0.01$

D-PDMP inhibits synthesis of lactosylceramide and glucosylceramide and decreases the cellular level of LacCer and GlcCer in human ASMC

As shown in Figs. 4 and 5 using 5 and 10 μmol/L of D-PDMP alone we could inhibit Glucosylceramide levels in the cells at 24 and 48 h. For lactosylceramide also a decrease in levels was seen with 5 and 10 μmol/L of D-PDMP alone at 24 h, but not so much at 48 h. When cells were exposed to 1% and 5% D-PDMP coated biopolymer wafers for 24 h, we observed a dose-dependent decrease in the cellular levels of LacCer and GlcCer.

Surprisingly, sirolimus alone and 1% sirolimus-coated biopolymer wafers also dose-dependently inhibited the cellular level of LacCer and GlcCer, but not to the same extent as D-PDMP. When the incubation period was extended to 48 h, sirolimus alone did not inhibit LacCer and GlcCer levels. In fact, 5 and 10 μmol/L sirolimus increased LacCer levels. In sharp contrast, at 48 h, D-PDMP alone continued to inhibit LacCer and GlcCer levels. A pronounced and dose-dependent and highly statistically significant decrease in LacCer and GlcCer levels was observed in cells exposed to 1% and 5% D-PDMP coated biopolymer wafers ($p<0.05$)

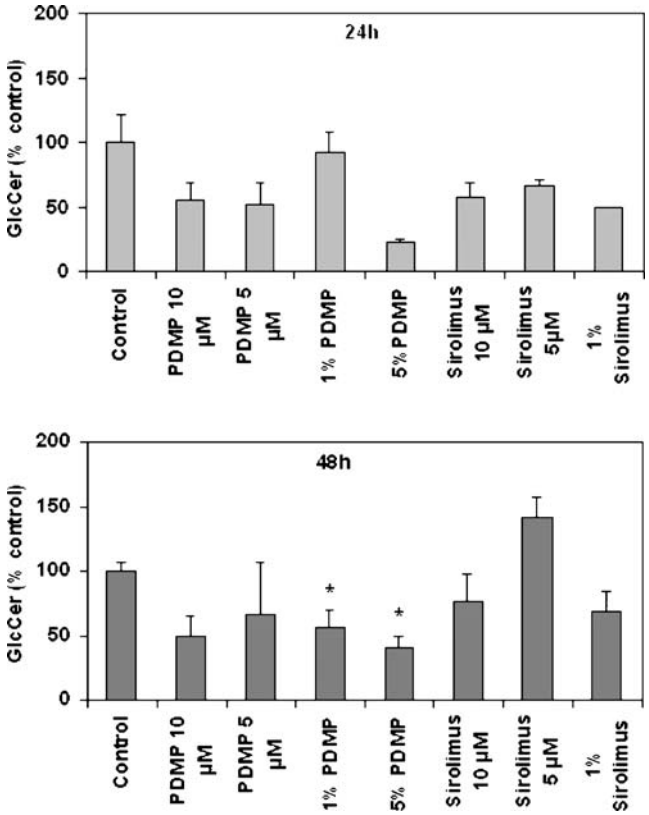

Fig. 4 Effect of D-PDMP and sirolimus on the glucosylceramide levels in human ASMC. Confluent cultures of human ASMC grown in six-well trays were incubated for 24 and 48 h with [^{14}C] palmitate (5 μCi/mL) and the following: PDGF (25 ng/mL) with control wafer, PDGF+sirolimus (10 μmol/L), PDGF+sirolimus (5 μmol/L), PDGF+ PDMP (10 μmol/L), PDGF+PDMP (10 μmol/L and 5 μmol/L), PDGF+1% PDMP wafers (18.98 μg/cm^2; 10 wafers/well), PDGF+ PDMP wafers (92.12 μg/cm^2; 10wafers/well) and PDGF+1% sirolimus wafers (18.98 μg/cm^2; 10 wafers/well). Next, the wafers were removed, cells were washed with PBS and extracted with hexane–isopropanol (3:2 by volume). The lipid extracts were dried in N_2 and subjected to silicic acid column chromatography. The glycosphingolipid fractions were separated further by HPTLC. Following autoradiography, gel area corresponding to glucosylceramide were excised and radioactivity was measured in a Beckman scintillation spectrometer. The data is presented as percent control (3,159±18.27 and 5,712.84±118.16 referring to cells incubated with PDGF±SD at 24 and 48 h respectively). *$p \leq 0.05$

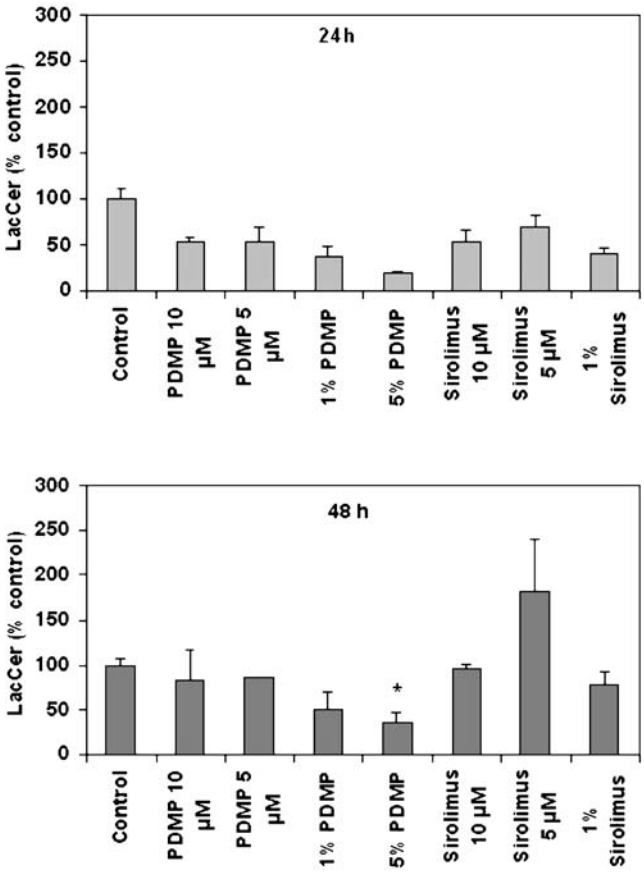

Fig. 5 Effect of D-PDMP and sirolimus on the lactosylceramide levels in human ASMC. Confluent cultures of human ASMC grown in six-well trays were incubated for 24 and 48 h with [^{14}C] palmitate (5 µCi/mL) with the same substrates and procedure indicated in legend to Fig. 4. Following autoradiography gel area corresponding to lactosylceramide were excised and radioactivity was measured in a Beckman scintillation spectrometer. The data is presented as percent control (3,707.14±69.67 and 5,092.4±421.6 referring to cells incubated with PDGF±SD at 24 and 48 h respectively). *$p \leq 0.05$

Angiographic studies in a rabbit model with bare metal stents and D-PDMP coated stents

Among the 12 stented rabbits, one rabbit died shortly after surgery, due to incomplete stent expansion in artery; other 11 rabbits were successfully implanted with either metallic or coated stents.

Angiogram results show that the stents were placed 4–5 cm above the cutting site and were fully expanded. There was no immediate occlusion after stenting as shown in Fig. 6. Rabbits recovered very well after surgery. Four rabbits subjected to angiography before sacrifice showed that the arteries were still patent after stenting for 2 weeks. All 11 rabbits survived for 2 weeks (longest period of study) and maintained good health. There were no sepses. Severe bleeding, diarrhea, anorexia, significant weight loss or other abnormalities were also not observed. Based on these observations, the D-PDMP coated metallic stents are safe and show good compatibility *in vivo*.

Discussion

Restenosis of blood vessels continues to be a major limitation for the long term successes of percutaneous transluminal coronary angioplasty (PTCA). Restenosis occurs as a result of many factors including vessel recoil, vascular remodeling, and residual plaque burden and neointimal hyperplasia [47]. Neointimal hyperplasia begins with the initiation of inflammatory response in the injured blood vessels leading to smooth muscle cells migration and proliferation and subsequent deposition of extra cellular matrix [28, 48]. PDGF was shown to play a major role in mediating the proliferative response of vascular smooth muscle cells [28]. Sphingolipids and their metabolites serve as lipid second messengers in mediating the proliferative effects of PDGF and other compounds such as VEGF, ox-LDL and TNF-α in evoking several phenotypic changes that contribute to atherosclerosis [37, 39]. One such sphingolipid metabolite, lactosylceramide functions as a key molecule involved in PDGF induced proliferation of vascular smooth muscle cells [34–36]. Therefore, LacCer could prove to be a potential target for pharmacological interventions to prevent restenosis.

In this study we explored the viability, *in vitro* efficacy and *in vivo* feasibility of using a stent coated with D-PDMP (inhibitor of GlcCer synthase and LacCer synthase) loaded biodegradable PLGA polymer.

Our study describes the following aspects of the D-PDMP coated stent usage: (1) *In vitro* drug release, material degradation. (2) Effect of the released D-PDMP on proliferation and programmed cell death of cultures of human ASMC. (3) Safety and bio-compatibility of using these stents *in vivo* by a short term trial in rabbits. Comparison of the *in vitro* cellular effects of D-PDMP (both in polymer coated form and alone) with sirolimus; an immunosuppressant widely used in DES was conducted simultaneously.

Our *in vitro* drug release experiments show that a sustained release of D-PDMP could be obtained for more than 8 weeks and thus sufficient levels of the drug could be delivered easily using these stents to exert an antiproliferative effect (Fig. 1a). The drug release pattern of D-PDMP from the PLGA coated stents was similar to that seen with curcumin loaded PGLA [45] coating mainly involving an initial burst of release followed by a more sustained release over the next few weeks. A comparison of molecular weight loss of the PLGA coating, measured using gel-permeation chromatography, revealed that D-PDMP causes faster degradation of PGLA in the *in vitro* medium

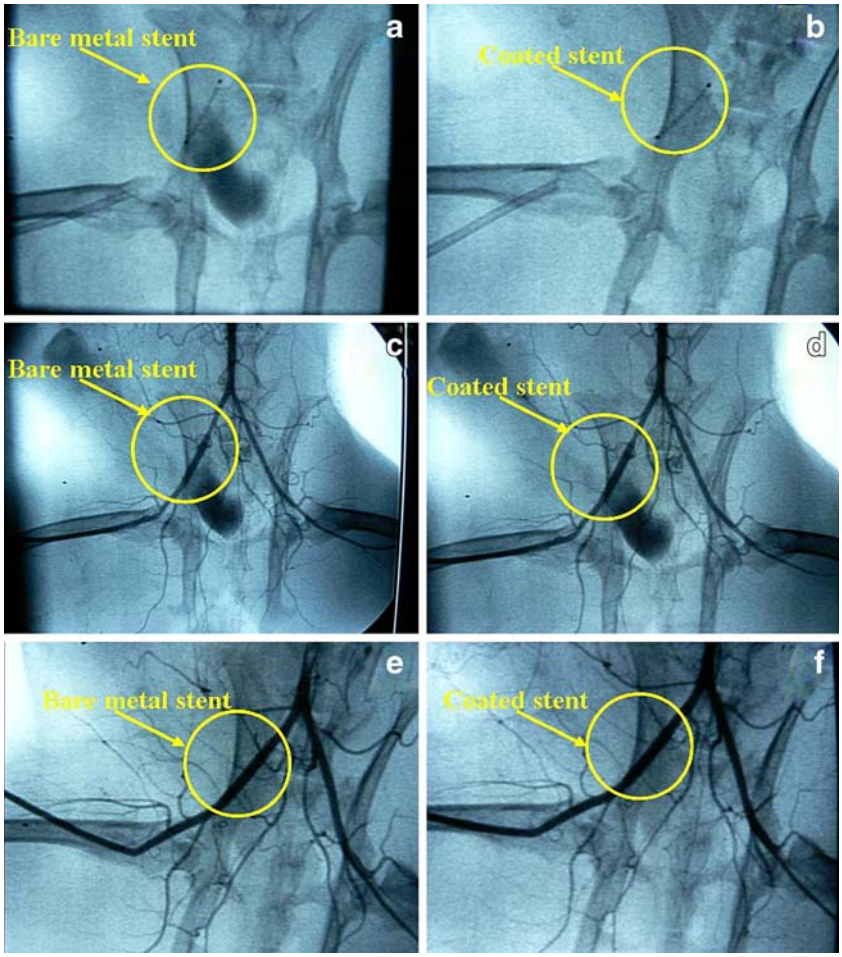

Fig. 6 Angiograms of the bare metal stent during stent insertion (**a**); after stent placement on day 1 (**c**); on day 12 (**e**). Angiograms of the 10% D-PDMP coated stents: during stent insertion (**b**); after stent placement on day 1 (**d**); on day 12 (**f**)

compared to the controls (Fig. 1b). Overall, a sustained release of D-PDMP from these PLGA-coated stents for a long span of time may provide with enough drug concentration to prevent cell proliferation.

Having shown a sustained drug release from the stent we examined the *in vitro* effects of these polymer coated drugs on human ASMC and compared it with the effects of sirolimus. In human ASMC cultures a dose-dependent elution of D-PDMP was seen from the drug coated wafers at 24 h and at 48 h (data not shown). D-PDMP released from PLGA wafers as well as treating cells with D-PDMP alone inhibited PDGF-induced proliferation in human ASMC when incubated for 24 h (Fig. 2). A sustained inhibition was observed upon continued incubation up to 48 h with D-PDMP alone and 5% D-PDMP/PLGA whereas, 1% D-PDMP coated wafers no longer had this effect (Fig. 2). This may be explained due to a higher level of sustained release of the drug from 5% D-PDMP wafers.

Following incubation for 24 h, sirolimus also showed significant inhibition of PDGF-induced proliferation. However, this was not observed in 48 h cell cultures (Fig. 2). Thus, D-PDMP exerted a relatively more sustained inhibitory effect on PDGF-induced cell proliferation as compared to sirolimus.

Sirolimus and D-PDMP act by two distinct mechanisms. Sirolimus prevents proliferation by binding to FKBP12 protein and then inhibiting mTOR protein which is critical in ribosomal protein biosynthesis, cell growth and proliferation [49]. During the time of cell cycle progression, mTOR causes both the phosphorylation and activation of $p70^{S6K}$ (s6 ribosomal protein kinase); a kinase implicated in cell proliferation [50]. mTOR is also involved in the activation of translation factors in particular, eukaryotic initiation factor 4E (eIF-4E) [51, 52]. Sirolimus inhibits downstream signaling by mTOR resulting in the inhibition of cell cycle protein up regulation in carotid artery smooth muscle cells and thus ultimately inhibiting cell division [53]. Also it can interrupt cell cycle progression by causing accumulation of P27kip1 cyclin kinase inhibitor and inhibition of Pub protein [54, 55], thus leading to cell cycle arrest.

On the other hand, the mechanism of action of D-PDMP entails inhibiting GlcCer synthase and LacCer synthase enzymes. These enzymes are involved in the step wise generation of glucosylceramide and lactosyl ceramide from

ceramide, respectively. And in particular we have shown that D-PDMP inhibits PDGF-induced activation of LacCer synthase [33] We have also shown that in human ASMC LacCer, but not GlcCer activates an "oxygen sensitive" signaling pathway that involves the production of free oxygen radicals, p21 Ras GTP loading, activation/phosphorylation of the kinase cascade including the mitogen-activated protein kinase Akt, c-*fos* and cyclin expression that collectively contribute to cell proliferation. And this is inhibited by pre-treatment of cells with D-PDMP [33, 36–39]. Additional studies using oxidized LDL, TNF-α and VEGF suggested that these compounds also activated LacCer synthase in human ASMC, and endothelial cells [31, 32, 38] contributing to an increase in cell proliferation, cell adhesion, migration and angiogenesis; phenotypic changes that are part and parcel of atherogenesis. DPDMP was reported to inhibit such phenotypic changes. Thus, LacCer synthase inhibition is an important target for intervention by which restenosis could be prevented. In our experiment we took one step further in this direction by showing that DPDMP released from PLGA wafers was equally and even more effective in preventing PDGF induced proliferation as compared to D-PDMP alone and also it had more sustained/long lasting effect on proliferation than sirolimus; a commonly used compound in DES.

We also examined/compared the effect of D-PDMP and sirolimus on apoptosis in cultured human ASMC in the presence and absence of serum in the tissue culture medium in our studies. D-PDMP did not induce apoptosis significantly in human ASMC irrespective of the presence/absence of serum (Fig. 3).

On the other hand, sirolimus markedly induced apoptosis in human ASMC cultures irrespective of presence or absence of serum. This observation is also in agreement with previous studies [56]. Previously, decreased levels of anti-apoptotic proteins through inhibition of nuclear translocation of NF-KB protein [57] or by activation of Fas and Fas ligand pathway have been suggested as the most likely mechanisms by which sirolimus increases apoptosis in endothelial cells and vascular smooth muscle cells [58]. Therefore, by virtue of not significantly affecting the apoptotic death of endothelial cells, D-PDMP (data not shown) would likely support re-endothelialization compared to sirolimus.

In order to confirm the mechanism by which D-PDMP coated PLGA wafers was inhibiting human ASMC proliferation we measured the cellular levels of LacCer and GlcCer. We found a dose-dependent decrease in the levels of LacCer and GlcCer with 5% and 1% D-PDMP coated PLGA wafers and this inhibitory effect was continued but not as significant up to 48 h (Figs. 4 and 5). These findings are consistent with our previous studies wherein, PDGF-induced increase in LacCer level was also mitigated by D-PDMP. Sirolimus on the other hand, did not significantly affect the levels of LacCer and GlcCer over the duration of this study. These studies suggest that there is a dichotomy in regard to the mechanisms of action of D-PDMP as compared to sirolimus.

Our *in vivo* safety and biocompatibility studies using the angiograms from the rabbits implanted with the stents after 2 weeks of the intervention (Fig. 6) showed patent arteries and the absence of any complication such as sepsis bleeding, anorexia. These observations suggest that D-PDMP coated metallic stents are safe and show good compatibility *in vivo*. This opens the possibility of using these double layer coated stents with D-PDMP safely in animal models to further evaluate their *in vivo* efficacy in preventing neointimal hyperplasia.

In sum, D-PDMP coated on PLGA stents is efficiently eluted and its level maintained highly significantly for sustained periods of time (up to 8 weeks). Moreover, our *in vivo* studies reveal that such D-PDMP coated stents can withstand the rigors of bending and stretching. Our *in vitro* studies reveal that the polymer coated D-PDMP was equally or even more efficacious in preventing the PDGF proliferation as compared to sirolimus. We believe that the lack of apoptotic effect of D-PDMP would foster/facilitate re-endothelialization and early healing of the wound with decreased inflammation.

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