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Activation of Hypoxia-Inducible Factor by Di-Methyl Oxalyl Glycine (DMOG) Increases Neovascularization within Ischaemic Myocardium in a Porcine Coronary Artery Occlusion Model

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Abstract

Introduction: Chronic total coronary artery occlusion (CTO) in man remains a significant challenge for percutaneous coronary intervention (PCI) and a common reason for coronary artery bypass surgery, as often the CTO cannot be crossed. This study investigated whether a potential angiogenic treatment, the prolyl-4-hydroxylase inhibitor, di-methyl oxalyl glycine (DMOG), would increase collateral vessel formation and myocardial perfusion without opening the CTO in a porcine model of coronary CTO.

Methods: We assessed the effect of DMOG upon tube formation of HUVEC in a matrigel assay in vitro. DMOG was loaded onto a polymer-coated coronary stent. Copper-coated stents were used to produce a coronary CTO in 20 pigs. DMOG-loaded or control stents were implanted in an alternating, blinded manner at day 28, proximal to the CTO. Angiographic and physiological flow data were collected at day 56, when the animals were sacrificed and the collateral vessels counted.

Results: DMOG increased tubule formation in vitro by 77% compared with control ($p<0.0001$). DMOG was successfully loaded onto the polymer coated stent, as evidenced by the same assay. A CTO was present at angiography in all animals 28 days. At 56 days there was a trend towards a greater increase in angiographic collateral vessel area around the CTO in the DMOG group compared with controls +84.5% vs. +16.5%, respectively, $p=0.057$). Histology revealed a significant increase in the number of collateral vessels around the site of occlusion in the DMOG group vs control (29.9±2.6 vs 18.4±3.1, respectively; $p=0.01$). There was no difference between the groups in terms of collateral flow index at day 56.

Conclusion: DMOG, delivered on a polymer-coated stent, proximal to an occluded porcine coronary artery, increased the number of collateral vessels seen at the site of vessel occlusion but not to a level sufficient to increase measures of functional flow.

Keywords: Coronary stent; Coronary artery occlusion; Porcine model; Angiogenesis

Introduction

Chronic total coronary occlusion (CTO) is a frequent finding in patients with angina, with detection in up to 30% of patients undergoing coronary angiography. At most the average success rate for the most experienced operators in opening CTOs by percutaneous coronary intervention (PCI) remains around 80%, with a significant proportion of symptomatic patients requiring coronary bypass surgery [1].

Hypoxia inducible factor-1 (HIF 1) is a hetero-dimeric nuclear transcription factor induced in hypoxic cells which acts as a global regulator of oxygen homeostasis and angiogenesis. HIF-1 transcriptional activity is determined by expression and stability of the α subunit [2,3]. Under normoxic steady-state conditions hydroxylation of proline residues 402 and/or 564 by prolyl-4-hydroxylases leads to ubiquitination and proteosomal degradation resulting in low nuclear levels of HIF-1 [4,5]. Mice genetically modified to be homozygous null for the HIF-1α allele die in utero as a result of failed vascularisation [6]. A number of pre-clinical studies have been performed to investigate the effect of HIF-1 upregulation. Whereas local delivery of vascular endothelial growth factor (VEGF) results in development of capillaries exhibiting excessive permeability, transgenic expression of HIF-1α within mouse skin produces physiologically competent capillaries, suggesting better co-ordination of angiogenesis [7]. This observation is supported by the fact that HIF-1 has been shown to regulate multiple genes involved in angiogenesis and that adeno-virus mediated direct introduction of HIF-1 can induce angiogenesis in non-ischaemic tissue [8].

Di-methyl oxalyl glycine (DMOG) is a non-specific inhibitor of prolyl-4-hydroxylase (PHD) enzymes which 'tag' DMOG for ubiquitination and proteasomal degradation under conditions of

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normoxia. PHD enzyme inhibition has been shown to increase levels of HIF in cultured human endothelial cells [9]. We have previously shown that DMOG is active in-vitro after eluting from a polymer coated stent, and at a local concentration of 125μM DMOG promotes formation of tubule-like endothelial structures in matrigel culture [10]. Upregulation of HIF-1 has been shown in murine models to reduce ischaemic myocardial injury and reduce post-infarction negative remodelling [11,12]. Stabilization of HIF-1 by di-methyl oxalyl glycine (DMOG), a potent PHD inhibitor, has been shown to induce angiogenesis in ischaemic murine skeletal muscle [13], but no published data exist on the action of PHD inhibitors on cardiac angiogenesis.

Our hypothesis was that local delivery of DMOG would increase antegrade collaterals through increased local levels of HIF-1. We therefore aimed to study the effect of DMOG upon angiogenesis in a matrigel model in vitro, and then perform a blinded, controlled, investigation into the effect of DMOG upon collateral coronary vessel formation in vivo using a recently developed porcine endovascular model of CTO. The delivery device was a coated coronary stent and we used angiographic, physiological and histological end-points.

Materials and Methods

In vitro matrigel tube-formation assay

A sample of EHy926 cells, a HUVEC-derived endothelial cell line, was supplied by Professor A. Goodall, University of Leicester. They were cultured in a pyruvate-free high glucose Dulbecco's modified eagle medium (Invitrogen, Gibco 41965) with 2% HAT solution (Gibco Cat No. 21060), 2% Penicillin/ Streptomycin and 2% Fetal bovine serum (Amersham).

The primary culture medium was reduced-growth factor (RGF) matrigel (a matrix-rich product prepared from the Englebreth-Holm-Swarm tumour cells whose primary component is laminin [14,15]. To 24 inner wells of a 96-well culture plate was added a thin layer of 50ul RGF Matrigel (BD Bioscience) equivalent to 1.76μl/mm^2. 20,000 Ehy926 cells were added giving a cell density of 556 cells/mm^2 (well surface area 36mm^2). A top layer of 100μl of the standard cell culture medium was added after each cell inoculation. The wells were inspected by light microscopy to confirm even cell coverage.

A known concentration of one of the study compounds was then added to each well. Control wells were treated with glycine 250μM. A specific VEGF receptor 2 inhibitor (Amersham) was added at a concentration of 160μM to alternate wells. Culture media and study compounds were refreshed every 24 hours.

The plates were imaged with a 25x inverted fluorescent microscope (Axiovert, Zeiss) with and without addition of Dil-Ac-LDL, a specific endothelial cell marker. The images were captured by a digital imaging system (Olympus AZ500) linked to a PC running C14 image analysis software (Apple, inc) for subsequent off-line blinded quantitative analysis. Using the grid-overlay function of Image J (US National Institutes for Health, Bethesda, MD) with both manual and automatic edge-detection functions, the area covered by any tubule-like structure was determined. Additionally the number of 10x10 grid squares in each well containing a branching tubule-like cellular structure (tubule coverage percentage,%) was recorded.

In vivo study protocol

The *in-vivo* experiments were conducted in 20 animals. A CTO was induced in the right coronary artery (RCA) by implantation of a copper-coated stent at day 0 (see below). At day if a 28, when a mature CTO was present, the animals were randomized to implantation of either a DMOG-loaded, polymer-coated stent or a control polymer-only stent, the endpoint being measure of collateral vessel formation at 56 days (Figure 1). Treatment allocation was blinded to the investigators until data analysis had been completed.

Stent loading and elution

DMOG was supplied by the Chemistry Research Laboratory, University of Oxford and loaded into a polymer prior to coating the coronary stents. A programmable elution profile (PEP™, Lombard Medical, Sheffield, UK) polymer was used. This is a co-polymer of poly(vinyl pyrrolidone-co-vinyl acetate (PnVPA) and poly(vinylbutyral-co-vinyl alcohol-co-vinyl acetate (PVB) designed to allow release of DMOG over 28 days [16]. The polymer/DMOG mixture containing a dose of 400μg DMOG was spray coated onto S7 stainless steel stents (Medtronic Inc; Minneapolis, MN) size 2.75mmx 18mm (surface area of 77.83 mm^2). This dose was chosen as the maximum dose that could be loaded onto the polymer without polymer cracking. Direct physical detection of DMOG using mass spectrometry was unreliable due to the low molecular weight of DMOG (MW=175). A bio-assay approach was adopted for elution testing. Stents were placed on a hook and suspended in a vial containing 8ml of 40% human serum albumin solution with a 5mm magnetic flea. The vials were placed on a multipoint stirrer set at 100 rpm and incubated at 37°C for up to 30 days. Stent fragments were tested in co-culture with human umbilical vein endothelial cells (HUVEC) in a matrigel angiogenesis assay and the effect upon tubule-formation noted. Comparison was made with data from previous DMOG matrigel experiments [10].

Porcine copper stent model of CTO

We used an endovascular, porcine model of coronary artery CTO to test the effect of DMOG upon collateral vessels growing around the CTO. Our model is a variant of that described by Song et al, and employs the deployment of a copper-coated stent to elicit the development of a CTO [17]. The basis of the copper stent device was a stainless steel, balloon-expandable Bio*divYsio* stent, formerly manufactured by Biocompatibles Ltd, (Farnham, UK). The phosphorylcholine coating

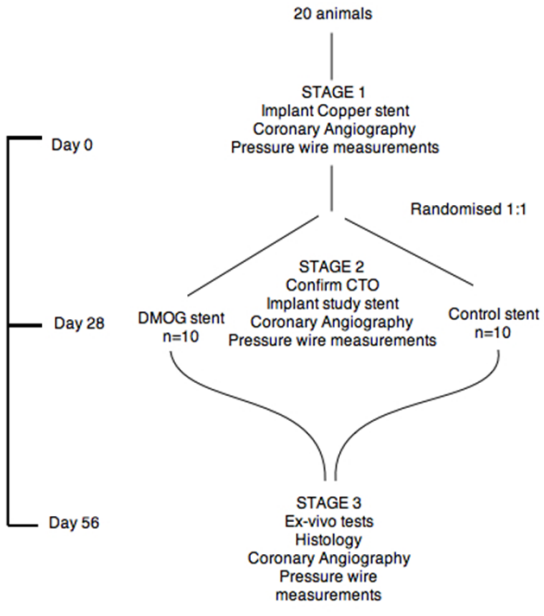


Figure 1: Study protocol.

was removed, a nickel coating was applied as a base layer and then the surface was electroplated with copper in a bath of nickel chloride. Electroplating was applied only to the abluminal aspect of the stent which was then re-sterilized.

Interventional procedure

Experimental procedures were performed according to UK Home Office regulations incorporating Local Ethics Committee approval [Dr J Gunn, Project Licence 40/2851; Personal Licence 50/2659]. Yorkshire White pigs were used, weighing approximately 30kg. Dual anti-platelet therapy (aspirin 37.5mg and clopidogrel 37.5mg daily) was administered for 7 days prior to the first intervention and continuing throughout the duration of the experiment on an empirical basis to reduce the risk of acute stent thrombosis. For each intervention (1,2 and 3) sedation was initiated with intramuscular azaperone and general anaesthesia was induced with intravenous propofol and maintained with spontaneously inhaled enflurane and oxygen. Vascular access was gained via the right internal carotid artery and, under fluoroscopic guidance, the left coronary artery was intubated with an Amplatz right, and the right coronary artery with a Judkins left, guide catheter. All interventional procedures were performed on the dominant RCA. Coronary angiography was performed in LAO 35° and RAO 30° projections. A coin was used for calibration. The table height was fixed at 27.5cm from floor level with the intensifier positioned 22cm from the subject to ensure constant magnification. Images were acquired with an image intensifier and digitally archived as Dicom files using via a Philips Xcelera™ workstation. Following the first procedure the right internal carotid was ligated, and it was possible in all cases to gain access to the RICA more proximally during the second procedure with no clinical sequelae. For the third (terminal) procedure at day 56 the left internal carotid was used for vascular access.

Flow measurements

Estimation of collateral flow was made at baseline (day 0) prior to copper stent implantation, at day 28 prior to study stent implantation and at day 56 prior to sacrifice.

A 0.014-inch pressure monitoring guidewire (PrimeWire, Volcano, San Diego, Ca) was used to calculate the collateral flow index (CFI). The pressure wire was calibrated in the guiding catheter. Aortic pressure was measured via the guiding catheter using a CardioMed Flowmeter system (CardioMed A/S, Oslo, Norway). Pressure wire measurements were transduced and recorded via a WaveMap (Endosonics Corp, Rancho Cordova, Ca.). Coronary pressure readings were recorded using the pressure wire. Mean aortic pressure (Pa), coronary distal pressure (Pd) and coronary wedge pressure (Pw) were recorded both at baseline and after a bolus intra-coronary (IC) injection of adenosine 60μg to produce maximal hyperaemic vasodilatation of the coronary capillary bed. Right atrial pressure (Venous pressure, Pv) was measured via a 6F diagnostic catheter after cannulation of the internal jugular vein. During experiments on day 28 no attempt was made to cross any coronary occlusion that was present after implantation of the copper stent to avoid disruption of the occlusive tissue. A technique of proximal balloon occlusion was used to provide a surrogate measure of Pw.

Stent implantation

Copper-coated stents were implanted at day 0 at a stent:artery ratio of 1:1 in the RCA. Quantitative angiography (CAAS, PIE Medical Imaging BV, Maastricht, Netherlands) was used to identify segments of the LAD and/or RCA that were of appropriate size. Standard angioplasty techniques were used. At the end of the procedure, the angioplasty equipment was withdrawn, the carotid artery ligated at high level and the animal allowed to recover. At 28 days, angiography and the pressure wire measurements were repeated. The study stent (DMOG or control) was then implanted immediately proximal to the copper stent at a stent:artery ratio of 1:1. At 56 days, the angiographic and pressure wire measurements were repeated. The animals were then sacrificed using intravenous pentobarbitone, and the stented segments of artery excised and processed.

Analysis of collateral vessels and flow

Digital acquisitions were stored as Dicom files and analysed off-line. Two indirect measures of collateral flow were assessed at day 28 and day 56. Direct assessment of the volume of collateral vessels was performed. Collateral volume was assessed using the Image J program, as above. Using a standard magnification a grid was overlaid on the angiogram such that each square had a diameter of 0.8mm, area 0.66mm^2. Each square that was completely filled by a collateral vessel that formed part of a complete bridging antegrade collateral was marked and the volume of the antegrade collateral network thus calculated.

Ex-vivo procedures

***Ex-vivo* assessment of coronary flow:** The heart was excised en bloc. A coronary infusion apparatus was improvised by suturing a 14G intravenous cannula around the RCA os, taking care not to occlude the artery. To this was attached an intravenous giving set and a 500ml bag of 0.9% saline. The saline bag was suspended at a fixed height of 4 feet (122 cm). Infusion was commenced and the proximal insertion site checked for leaks. The heart was placed on a set of electronic balance scales and the scales reset to zero. The infusion was started at its maximum infusion rate and the time taken for the scales to register 100g (i.e. 100 ml saline) was noted. From this a standardized coronary flow rate (ml/min) was estimated.

Histological processing: The coronary arteries were then carefully dissected free with a minimum of surrounding tissue and flushed with saline. The two adjoining stents (study stent most proximal, copper stent distal) were identified. The artery was opened as a flat sheet preserving the stent. Sections were taken as follows: 2 from proximal to the study (DMOG or control) stent; 2 around the study stent; 2 around the copper stent; and 2 distal to the study stent. Further sections were taken from the mid left anterior descending artery to act as untreated controls (Figure 2). The tissue samples were labelled and stored in formalin for subsequent staining with haematoxylin and eosin and processing for immunohistochemistry. After processing the tissue samples, copper stent sections were excised and fixed in 10% formalin, embedded in T8100 resin, cut with a diamond-tipped saw and ground and polished to produce multiple cross-sections. Sections were stained with hematoxylin and eosin (H&E) and prepared for microscopy. This histological preparation has been described previously by our group [18]. Staining for endothelial cell origin was undertaken by staining for CD-31 antigen and von Willebrand factor. Slides stained with H&E were photographed using a Zeiss Axiovert microscope at 40x magnification and Olympus digital camera with C14 image analysis software. To assess the number of collateral vessels present, the central lumen of the RCA was first identified at low power. The total number of micro-vessels was counted in 40 high power fields (10 high power fields in each of 4 quadrants centred on the RCA lumen). This process was repeated for both sections in each sampling position and an average value obtained. Micro-vessels were measured and the number of large (≥100 μm) microvessels recorded.

Statistical analysis

No formal a priori sample size calculation was possible given the exploratory nature of this work and the use of a novel CTO model. Continuous data was compared using independent 2-tailed Student's t test. Analysis was performed using Prism 4 (GraphPad Software, Inc.)

Results

In vitro HUVEC tubule-formation model

The extent of tubule formation was increased by the addition of DMOG at a concentration of 100-200μM. At 24 hours, maximal tubule formation was seen with DMOG 125μM. At concentrations greater than 200μM DMOG there was a gradual reduction in the extent of tubule formation with concentrations greater than 500μM inhibitory (Figure 3). There was no tubule formation before 8 hours in the HUVEC/matrigel tubule formation assay. Tubule formation was complete between 24 and 36 hours. Tubule formation was significantly increased by addition of DMOG 50μM although the magnitude of the increase was small. DMOG 125μM increased tubule formation compared with glycine control (tubule coverage 75.6% vs. 42.7%, $p<0.0001$) and to a similar extent as VEGF 25μg/ml solution (Figure 3). The pro-angiogenic effects of both DMOG and VEGF were abolished by addition of VEGF 2 receptor inhibitor. Reproducibility of results was good using 12 wells per compound.

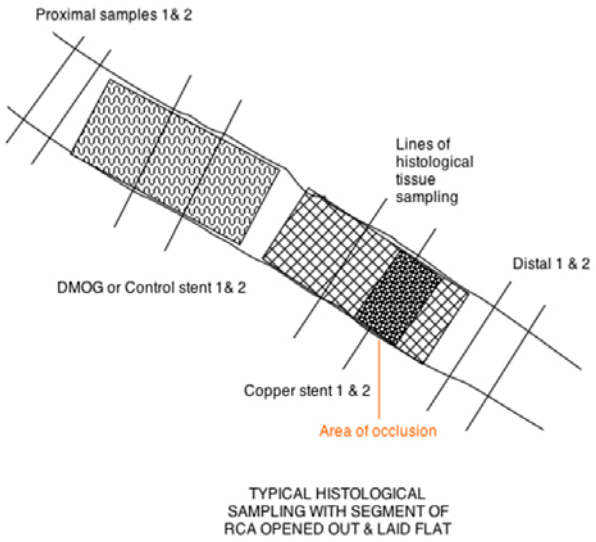
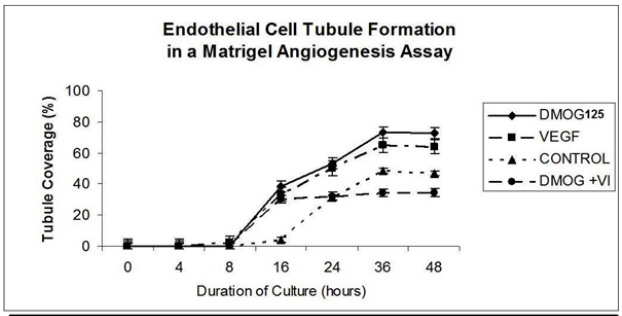


Figure 2: Location of histological sampling.



Tubule formation [mean (SEM), tubule coverage%] n=12 wells						
Compound	**8hrs**	**16hrs**	**24hrs**	**36hrs***	**48hrs**	**p***
Glycine	0.2 (0.6)	4	33.8 (3.2)	42.7 (3.2)	42.1 (3.1)	
DMOG 50um	0.1 (0.3)	8.4 (2.9)	43.2 (4)	50.3 (5)	50.3 (5.5)	0.002
DMOG 125um	3 (1.6)	38.7 (3.6)	52.8 (3)	75.6 (3.3)	75.1 (2.9)	<0.0001
DMOG 125uM + VI	0	30.3 (2.5)	31.9 (5)	32.5 (3)	32.1 (3)	<0.0001
VEGF	0	33.4 (4.6)	50.7 (2.4)	70.1 (4.7)	69.9 (4.5)	<0.0001
VEGF + VI	0	0. 6 (1.1)	26.1 (4.1)	33.9 (3.2)	33.7 (3.2)	<0.0001

Figure 3: *In-vitro tubule-formation model.*Tubule coverage%, a measure of tubule coverage. VI= VEGF2 receptor inhibitor 160μM. SEM= Standard error of mean.

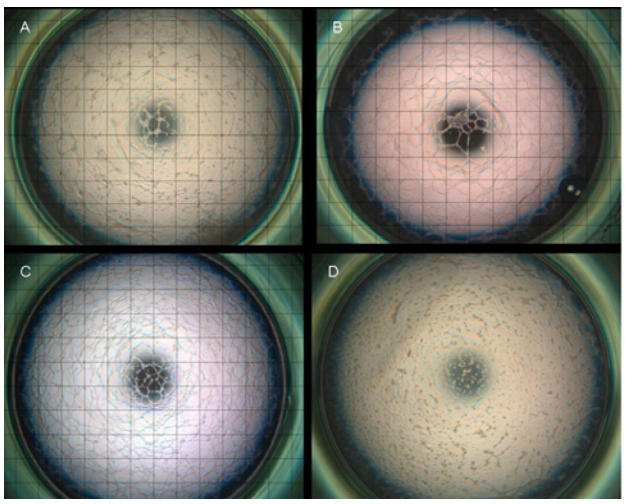


Figure 4: *In-vitro HUVEC tubule-formation model*- images at 36 hours. (A) Control, 38% tubule coverage (B) VEGF 69% tubule coverage (C) DMOG 125uM 74% tubule coverage (D) DMOG 1mM, minimal tubule formation.

Stent loading and elution

There was increased tubule-forming effect in the matrigel bio-assay with addition of stent fragments after elution in albumin solution for up to 21 days. The effect was similar to that observed with a concentration of 100-250μM DMOG direct to endothelial cells in culture.

Interventional procedure

20 animals underwent implantation of a copper stent. 17 animals (85%) survived to 56 days and completed the study. One animal in the control group died on day 1 following copper stent implantation from acute stent thrombosis. There was a complete vessel occlusion in all 19 surviving animals following copper stent implantation. All these animals had angiographically visible bridging antegrade collateral microvessels. The majority of these vessels appeared angiographically to occupy the adventitia surrounding the CTO. Two animals died in the DMOG stent group shortly after implantation of the second study stent; one had evidence of acute thrombus in the left anterior descending artery, and the other had no coronary occlusion but atrial thrombus was found to be present.

Angiographic collateral vessel area

The CTO present in all animals at 28 days following copper stent implantation exhibited extensive antegrade collateralization (Rentrop grade 4, Figure 6). There was no difference in the absolute collateral

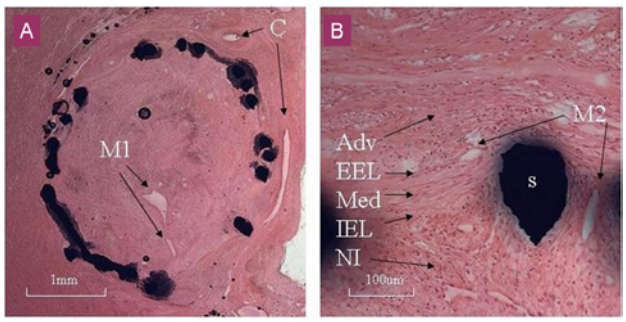


Figure 5: (a) Section from a pig coronary at 28 days (hematoxylin and eosin). The neointima is mature, and completely occludes the lumen. 'Large' microvessels (100-200μm diameter) are visible in the centre of the occlusive neointima (M1) and similar sized collateral vessels are seen in the adventitia around the stented segment **(C)**. **(b)** High power view of the neointima around a stent strut from another animal at 28 dyas. The neointima (NI), internal elastic lamina (IEL), media (Med), external elastic lamina (EEL) and adventitia (Adv) are labelled. 'Small' microvessels (10-20μm diameter) are visible in the neointima around the strut. The neointima of the CTO appears to be composed of the typical vascular smooth muscle cells and matrix.

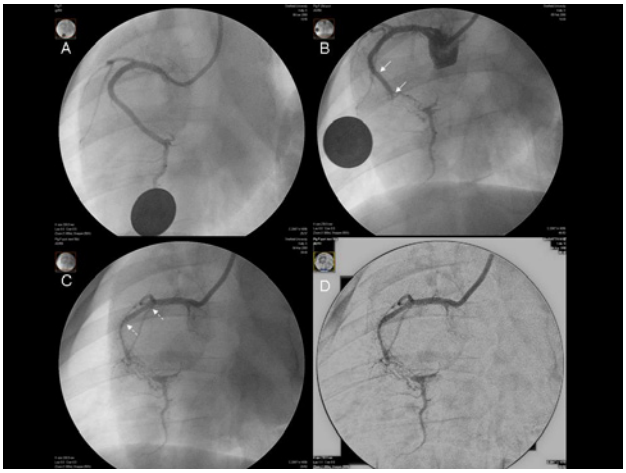


Figure 6: Angiographic images from a control group animal. A Initial RAO 30° view of RCA on day 0 prior to implantation of copper stent. B Same view at day 28 demonstrating mid vessel CTO at distal edge of copper stent (solid arrows) with antegrade bridging collaterals. C Day 56 following implantation of study (control, PEP polymer only) stent (dashed arrows). D Digital subtraction image of C used to highlight microvessels for image analysis.

vessel area at 56 days between the control and DMOG groups: 26.5 $mm^2 \pm 4.1mm^2$ vs 25.7 $mm^2 \pm 5.2$ mm^2, respectively; p=0.91. There was, however, a trend to a greater increase in visible collateral vessels following DMOG stent implantation with lower collateral vessel area in the treatment (DMOG stent) group prior to implantation ($15.8mm^2$ DMOG vs. $22.9mm^2$ control). The absolute increase in collateral area from day 28 to day 56 was $3.6mm^2 \pm 1.5$ mm^2 in the control group and $10mm^2 \pm 4.1$ mm^2 in the DMOG group, p=0.15. This was equivalent to an 84.5% increase in the collateral area among the DMOG group compared with the 16.5% in the control group. No retrograde collateralization was noted from the left coronary system.

Coronary physiological measurements

No significant difference was detected between the control or DMOG groups either before implantation of the study stent (at day 28) or on day 56: CFI 0.32±0.06 vs. 0.26±0.07 (units?), respectively; p=0.07.

Ex-vivo assessment of coronary flow

There was no difference in RCA flow rates between the control and DMOG groups: RCA 58.2±6.5 vs 58.4±6.6 ml/min, respectively; p=0.98.

Histological analysis of collaterals

In the region of the copper stents a moderately dense inflammatory cell infiltrate of leukocytes, monocytes and smooth muscle cells were noted. There were significantly more collateral micro-vessels seen around the copper stent (the area of occlusion) in the DMOG stent group vs control (29.9±2.6 vs 18.4±3.1, p=0.01). Collateral vessel density was similar in the proximal vessel and distal to the copper stent. There was no difference in the number of adventitial micro-vessels seen in segments of (control) mid left anterior descending artery from animals in either group. Immunohistochemistry confirmed neovascularization within the RCA sections around the copper stent predominately arising from within the adventitia, and positive staining of endothelial cells within these vessels for von Willebrand factor.

Discussion

The two main findings of our study were: an increase in neovascularization at 56 days around the site of copper stent implantation in those animals who received a DMOG coated stent, compared to control; and a failure of this to be reflected in increased physiological parameters. There was a statistically significant increase in histological micro-vessel density (number of collaterals per unit area) and a trend towards a greater increase in angiographic collateral volume around the site of the occlusion. There was no difference in histological micro-vessel density in the distal vessel between the DMOG and control stent groups, and consequently no difference in collateral flow index.

Previous clinical studies using protein growth factors such as VEGF or fibroblast growth factor (FGF) have been disappointing. Angiogenesis is complex, requiring numerous coordinated processes leading to the formation of physiologically competent blood vessels. Therapeutic use of a 'master-switch' compound that coordinates angiogenesis is therefore conceptually attractive. HIF-1α modification has been shown to induce neovascularization in a variety of pre-clinical models but has not been evaluated in cardiac tissue.

The effect of DMOG in this study must be interpreted in the context of the profound pro-inflammatory effects of copper on the vasculature: indeed an inflammatory cell infiltrate was noted in the region of the copper stents. The technique of CTO induction by copper-coated stent implantation is effective, and is accompanied by similar histological appearances to that of clinical CTO [17]. A notable feature of this model is the result development of generous antegrade collateral vessels with complete distal recipient vessel filling (Rentrop grade 3) and TIMI grade 3 flow in the distal vessel at 28 days following copper stent implantation, prior to implantation of the study stent. This collateralization was reflected in a significant increase in CFI following copper stent implantation. It could be, therefore, that the increase in collateralisation in both groups simply due to the stimulus of the copper stent effectively masked any additive effect of DMOG

Measurement of collateral flow index using the intra-coronary pressure wire is a well validated measure of collateral function. We elected not to cross the occlusion at day 56 and rather relied on a surrogate method employing a proximal occlusion balloon. This method was consistent across all three stages of the experiment and

had the benefit of not disrupting the artery and affecting subsequent histological analysis. The approach of measuring coronary wedge pressure as an index of distal collateral flow is widely accepted [19,20] and has been used in clinical studies to assess antegrade collateral perfusion [21]. Our results, demonstrating no increase in CFI in the DMOG group compared with control, were consistent with no increase in distal microvessel density. Histological analysis was based on simple counting of collateral micro-vessels within a set number of high power fields. Although this approach cannot discriminate between pre-existing adventitial micro-vessels and new collaterals it had the advantage of being simple, reproducible and constant between the groups.

Our study has a number of limitations. Our sample size was relatively modest, limited by the complexity of the repeated interventions and observations required. We were unable to measure local or systemic levels of HIF-1 or DMOG. Previous studies have demonstrated significant upregulation of HIF in response to systemic DMOG [13] but the precise amount of DMOG delivered to the site of the CTO in the present study was not known. Local tissue hypoxia may be induced by vessel occlusion and lead to upregulation of HIF: however prior to use of the copper stent technique collateralization was thought not to occur in the pig [17]. The extensive collateral vessel formation we observed at 28 days post copper stent may have attenuated any pro-angiogenic effect of the DMOG. Furthermore our methods for measuring distal coronary flow may have been insensitive to subtle changes in myocardial perfusion and were unable to provide information on presence of hibernating myocardium. Newly described techniques such as ex-vivo micro-CT may provide more sensitive measures of histological microvessel density than light microscopy alone [22]. Whilst this model of CTO is highly effective and preferable to external compression as an analogue of spontaneous coronary occlusion, the extent to which the intense fibrotic inflammatory reaction affects the response to growth factors is unknown. The effect of copper-induced inflammation on local neovascularization is poorly understood and requires further clarification.

The finding of increased neovascularization around the area of the copper stent in the DMOG treated group, but not more distally, is intriguing and compatible with data from murine models showing increased skeletal muscle neovascularization with DMOG in ischaemic tissue but lesser angiogenic effect in areas of tissue normoxia [12]. Well developed antegrade collateral vessels seen following copper stenting are likely to have minimized the extent of ischaemia in the territory subtended by the CTO. In this study, DMOG appears to have exerted greatest effect in the region of the occlusion around the copper stent. The precise pharmacokinetics of DMOG release and distribution following release from the stent is unknown and it is possible that sub-therapeutic concentrations of DMOG were delivered to the distal circulation. The extent of baseline collateralization seen in this study, however, not only makes significant distal ischaemia unlikely but makes assessment of any additional downstream therapeutic effect on perfusion difficult.

In summary, DMOG delivered by a polymer-coated coronary stent implanted proximal to the CTO, has local pro-angiogenic effects at the site of coronary artery occlusion. This did not however translate into an increase in indices of downstream reperfusion. This may have been because the model itself has limitations, such as the formation of extensive baseline antegrade collateral vessels prior to DMOG administration. This study confirms the pro-angiogenic potential of HIF-1α upregulation as a potential future therapy for occlusive vascular disease.

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