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Intra-arterial Photodynamic Therapy Using 5-ALA in a Swine Model

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Objectives: *to test the hypothesis that intravascular light could be delivered via a balloon catheter for arterial photodynamic therapy (PDT)*
Design: *pig non-injury model*
Materials: *clinical catheter equipment.*
Methods: *large White pigs (15–20 μg) were photosensitised with 5-aminolaevulinic acid (5-ALA) induced protoporphyrin IX (PpIX) at a concentration of 120 mg/kg Arterial biopsies were taken at intervals between 30 mins and 24 h and frozen sections analysed using a CCD camera to give a temporal profile of fluorescence in each arterial layer. PDT was given to normal arterial segments via a 4 mm transparent PTA balloon inflated so as to occlude flow, but not distend the artery. Animals were culled at 3 and 14 days and the above segments harvested.*
Results: *fluorescence peaked in the adventitia, intima and medial layers at 1 5, 4 and 6 h respectively PDT at all time points produced VSMC depletion compared with controls. The degree of depletion mirrored the fluorescence profile of PpIX*
Conclusions: *PDT can be delivered via a standard PTA balloon with a transparent channel This depletes the VSMC population within the arterial wall without complications. Intra-arterial PDT is therefore a potential therapy to reduce the incidence of restenosis post-angioplasty.*

Key Words Photodynamic therapy; Angioplasty, Restenosis.

Introduction

During the last two decades, percutaneous transluminal angioplasty (PTA) has become established in the treatment of atherosclerotic coronary and peripheral arterial disease. Advances in catheter design and improved techniques have allowed more complex and more distal stenoses and occlusions to be successfully treated leading to excellent immediate "radiological" results. However, such results are, in some cases, shortlived as it is now accepted that 12–50% of all PTAs restenose within 6 months post-procedure.[1–4] In Holmes' report of follow-up angiography from the PTCA Registry,[5] the restenosis rate was found to be definition dependent, but the overall rate was 34% from centres with combined repeat angioplasty rate of 84% of all cases. Moreover, in cases which restenose, the majority do so within 3 months with a small additional number between 3 and 6 months.[6] Reported restenosis rates following peripheral angioplasty are probably equivalent, but the data is less accurate due to lack of angiographic follow-up.

The mechanisms behind the restenosis process are still not fully understood and it is therefore not surprising that currently there are no proven therapeutic options to prevent it in clinical practice. Much effort has gone into trying to prevent the intimal hyperplastic response to balloon injury and following Austin's post-mortem studies,[7] the natural target for such work has been the vascular smooth muscle cell (VSMC). In the porcine restenosis model it has been established that VSMC proliferation and matrix formation are the major contributors to neointima formation.[8] This was initially believed to be the sole mechanism of restenosis until more recent studies[9–15] in both animal and human studies have revealed a remodelling component. Remodelling describes changes in all vascular dimensions following injury which may be favourable where the vessel (and lumen) increase in size, or unfavourable where there is a reduction in the external elastic lamina as well as intimal hyperplasia resulting in lumen loss.[9] It is now established that remodelling contributes more to restenosis than neointimal hyperplasia following angioplasty alone, but since stenting limits the effects of remodelling on lumen area, restenosis following

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stenting is almost exclusively secondary to neointimal hyperplasia.[16] Up to 70% of coronary angioplasties are now stented, so a method of reducing neointima formation remains an important goal.

Photodynamic therapy (PDT) produces a tissue effect by a photochemical reaction (following the interaction of light and a photosensitising agent) which allows reactive intermediates to cause cellular death. It has already been established in work with tumours, that rapidly proliferating tissues show partial (but, not absolute[17]) selectivity in the uptake of photosensitiser and can therefore be targeted allowing relative sparing of normal tissues The magnitude and depth of this effect is known to be dependent on the type of photosensitiser, the light dose and the interval between the photosensitiser and the light.

Vascular PDT has been shown to be effective in depleting the VSMC population *in vitro*[18,19] and in small animal studies[20–24] using a variety of photosensitisers, without complications of thrombosis or aneurysm formation. However, the limitations of small animal models are now clear[25–27] and if vascular PDT is to be proposed as a therapy to prevent angioplasty restenosis in humans it must first be shown to be effective in a large animal model.

The development of a new photosensitiser, 5-aminolaevulinic acid (5-ALA) has allowed PDT to be applied to situations other than those requiring full thickness necrosis 5-ALA induces endogenous porphyrin sensitisation via an active metabolite, protoporphyrin IX (PpIX) which following interaction with red light produces cellular killing to a depth of 1mm. In the treatment of malignancy, 5-ALA's depth of penetration is a disadvantage, but for vascular PDT it would be suitable for treating the arterial wall while limiting damage to deeper tissue.

For vascular PDT to be a feasible adjunctive therapy to prevent restensis following balloon angioplasty, light would have to be delivered via an endovascular technique using a laser fibre delivering light through a balloon catheter. This study aimed to measure the pharmacokinetics of PpIX (the active metabolite of 5-ALA) in the arterial system and investigate the safety and efficacy of endovascular light delivery for PDT in a swine model.

Methods

Pharmacokinetics of 5-ALA

All animal studies were carried out under licence (Animals (Scientific Procedures Act) 1986) and with the cooperation of the named veterinary surgeon. Six Large White/Landrace crossbred pigs were anaesthetised with inhaled halothane following premedication with intra-muscular medetomidine hydrochloride at 10–20μg/kg. Animals were intubated and maintained under spontaneous respiration with a mixture of halothane, nitrous oxide and oxygen at 1.5%, 0.5 and 4l/min respectively. 5-ALA (Dusa, U.S.A.) was given in an intravenous preparation (buffered to pH 4.8 using 8.4% sodium bicarbonate) at a concentration of 120mg/kg in a bolus injection via an ear vein.

Animals were cleaned and draped under sterile conditions. In four animals sequential arterial biopsies were taken from time zero to 8h (at 30 min intervals to 2h and then hourly) and in two, 24 hour samples were taken following sensitisation the day before. Biopsies were taken via longitudinal groin and neck approaches for access to external iliac and carotid arteries respectively and were randomised with respect to biopsy site and time point between animals. One centimetre transverse sections of arteries were excised between ligatures, snap frozen in precooled isopentane and stored in liquid nitrogen. Care was taken to ensure that sequential biopsies were taken from progressively more proximal sites which remained perfused.

Three transverse frozen sections (10μm thick) were cut from each block and stored at −20°C before being thawed just prior to fluorescence microscopy. Fluorescence of PpIX was excited by a helium-neon laser at 633nm and the signal detected between 665 and 710nm by a cryogenically cooled CCD (charge-coupled device) camera fitted to the fluorescence microscope and linked to an IBM computer. False-colour images were generated and quantitative analysis performed by measuring the mean arbitrary pixel count for each arterial layer. Three sections per animal per time point were analysed and nine readings taken from each section; three from each of the adventitia, media and intima. Background counts were periodically checked to confirm adequate equipment cooling and autofluorescence from control (time zero) sections subtracted from each count. Mean counts for each layer were then plotted for each time point.

PDT of normal arteries

A further eight pigs were photosensitised with 120mg/kg 5-ALA which was given as a bolus intravenous injection via an ear vein whilst under sedation Once photosensitised, pigs were kept in subdued light until

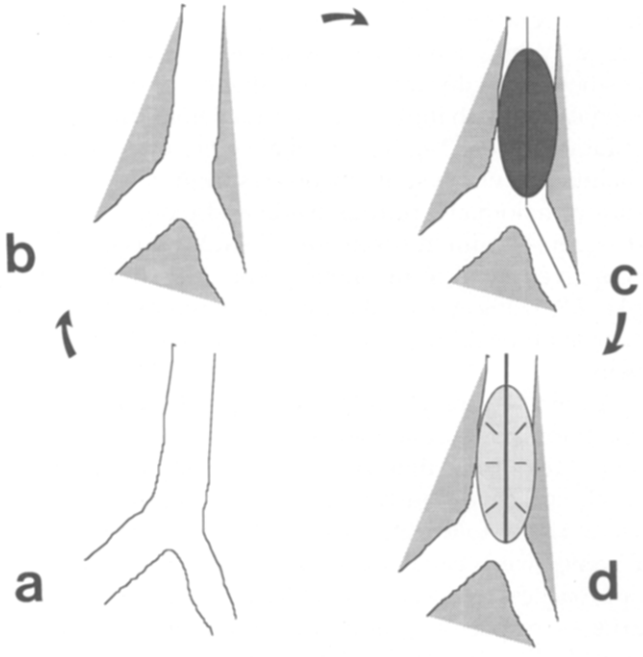

Fig. 1. Schematic diagram to illustrate intra-arterial PDT (a) Unsensitised iliac artery, (b) artery following 5-ALA, (c) balloon catheter over guidewire deployed within artery; (d) laser fibre substituted for guidewire, lighting sensitised arterial segment

anaesthetised, as before, and maintained with inhalational halothane and nitrous oxide. A 5 cm longitudinal incision was made in the left neck and the carotid exposed and controlled between slings. A 7FG sheath was introduced over a guidewire via an arteriotomy and Seldinger technique and 5000 IU of heparin given intra-arterially. A peripheral angiogram was performed with 10 ml of Omnipaque (350 mgI/ml) and stored on an image intensifier (Siemens) to produce a "road map", and under screening, a guidewire advanced into one common iliac artery. A 4 mm/4 cm transparent balloon catheter (Cordis, U.K) was advanced over the wire and the balloon inflated within the common iliac artery to 4 atmospheres (to occlude, but not distend the artery) and a further angiogram performed to confirm occlusion of the artery. The guidewire was then removed and exchanged for a laser fibre with a 4 cm 200 μm radial diffuser at its tip (Rare Earth Medical, U.S.A.) which was positioned within the balloon segment, as illustrated schematically in Fig. 1. Red light at a wavelength of 635 nm was generated from a copper vapour pumped dye laser (Oxford lasers) and 50 J per cm^2 of the surface of the inflated balloon given with a 60 second break in light delivery after 20% of the dose. During this fractionation period when the laser was off, the balloon was deflated to perfuse the limb. The irradiation time required to generate 50 J/cm^2 varied between 1350–1950 sec. Post-procedure, patency was checked with a further angiogram and the process repeated in the contralateral iliac artery.

Arterial segments were treated at 1.5, 2.5 or 6–7 h post-photosensitisation and control procedures – light alone (in an unphotosensitised animal) and balloon only (no light in a photosensitised animal) were performed. Following completion, the introducer sheath was removed, the carotid ligated and the skin closed. Animals were recovered and kept in subdued light for 24 h. Aspirin (300 mg) was given from 1 day preoperatively until culling.

Four animals were culled at 3, and 4 at 14 days postoperatively by a lethal dose of iv pentobarbitone. Control tissue was harvested at 3 days postoperatively. A bilateral retroperitoneal dissection was made to expose the treated iliac segments which were controlled with slings and clamped proximally and distally. A 20FG intravenous catheter was inserted via a constant side branch and the segment pressure perfused *in situ* with a 4% solution of formyl saline at 100 mmHg before excision. The excised artery was divided into proximal, middle and distal treated segments and stored for 16 hours in 4% formyl saline fixative. Two transverse histological sections from each segment were stained with H and E and examined by light microscopy (Nikon Labophot-2) to assess the number of VSMCs per high power field (HPF). Microscopy images were transferred onto a 486 personal computer via a colour camera (JVC TK-1281) and morphometric analysis performed using a Lucia-M (Version 3.52a) programme. Each section was imaged and the number of VSMCs per HPF counted in four fields per section at 12, 3, 6 and 9 o'clock. Counts for PDT treated segments were averaged and compared with controls

Sections from one block obtained at each treatment time point were stained with polyclonal Factor VIII (Dako, Denmark) using a Strep-avidin horse radish peroxide technique as a specific marker for endothelial cells. These were examined by light microscopy.

Statistical analysis between treatment subgroups and controls was by analysis of variance (ANOVA) using a Statview 4.5 computer programme (Abacus Concepts, California). Subgroups were compared using Fishers' PLSD post hoc test if the F-value was significant.

Results

Pharmacokinetics

False-colour images reveal high PpIX activity (highest intensity) in different arterial layers at different time

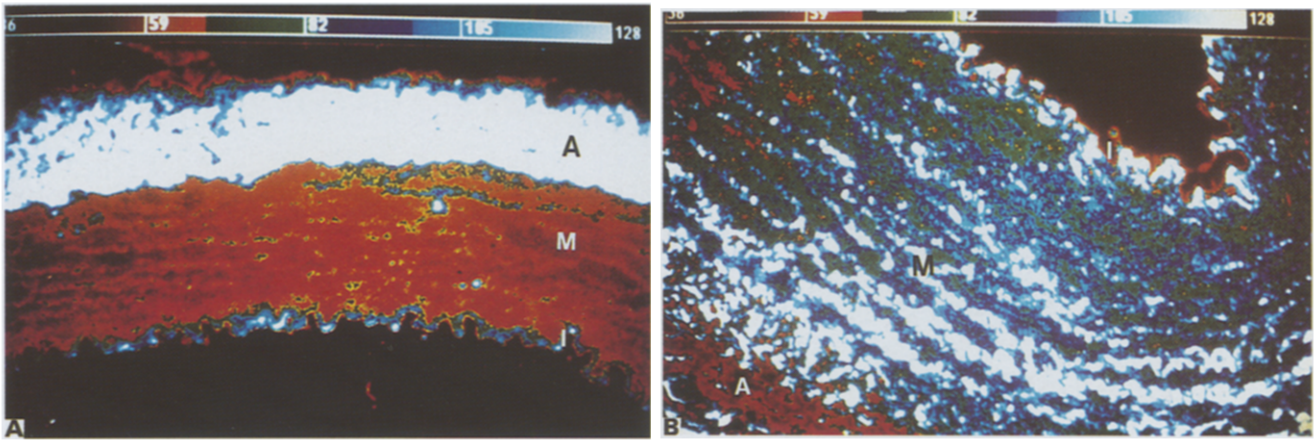

Fig. 2. False-colour fluorescence images of transverse sections through arterial biopsies taken (a) 1.5h and (b) 7h following 5-ALA administration. High adventitial uptake is noted at 1.5h which is reduced by 7h, when maximum fluorescence is seen in the media. L = lumen, I = intima, M = media, A = adventitia

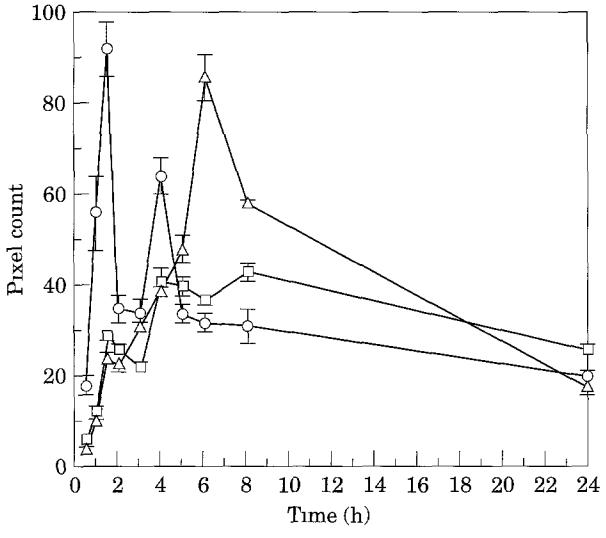

Fig. 3. Graph showing the temporal profile of fluorescence (expressed as a mean pixel count) for each arterial layer at time points 0 to 24h following 5-ALA administration at 120mg/kg. (□) Intima, (△) media, (○) adventitia

points (Fig. 2) PpIX activity as measured by fluorescence was seen to exhibit a double peak in the adventitia, initially at 1.5h and again at 4h. Fluorescence peaked in the media at 6h and showed a steady rise in the intima to 4h followed by a plateau from 4 to 8h (Fig. 3).

Histology and morphometry of PDT treated arteries

All animals survived to culling and there was no evidence of thrombosis, rupture or aneurysm formation in any of the PDT treated or control segments. A transverse section of a treated and control artery is seen in Fig. 4 showing depletion of the VSMC population in the segment which received PDT. Mean (±2 S.D.) VSMC counts per HPF were 115 (17) for control sensitised arteries; 103 (8) for laser alone treated unsensitised arteries; and 27 (29) ($p<0.0001$) for arteries harvested at either 3 or 14 days following PDT.

Comparing PDT at different time points (drug-light intervals) showed that the maximal effect (as measured by VSMC depletion in the media) was seen at 1.5 and 6–7h post sensitisation, corresponding with the pharmacokinetic profile of PpIX as seen in Fig. 3. From tissue harvested at 3 days, mean (±2 S.D.) VSMC/HPF in treatment groups, compared with controls, were reduced at all time points; 22 (19) ($p<0.0001$) at 1.5h; 51 (29) ($p<0.0001$) at 2.5h and 11 (10) ($p<0.0001$) at 6–7h as seen in Fig. 5, but depletion appeared to be most at times corresponding to the first adventitial peak and the later medial peak.

Arterial segments treated at the same drug light interval, but harvested either at 3 or 14 days were analysed separately (Fig. 6). Mean VSMC/HPF (±2 S.D.) from treatment groups harvested at 3 days (8 (12)) and 14 days (17 (19)) were significantly ($p<0.0001$) reduced compared with controls.

The presence or absence of endothelial cells was assessed using polyclonal Factor VIII as a marker which showed complete absence of endothelial cells from sections harvested at 3 days, but repopulation by day 14. Endothelial cell repopulation occurred early, but VSMC depletion persisted at 14 days and no appreciable neointimal hyperplasia occurred in treated sections.

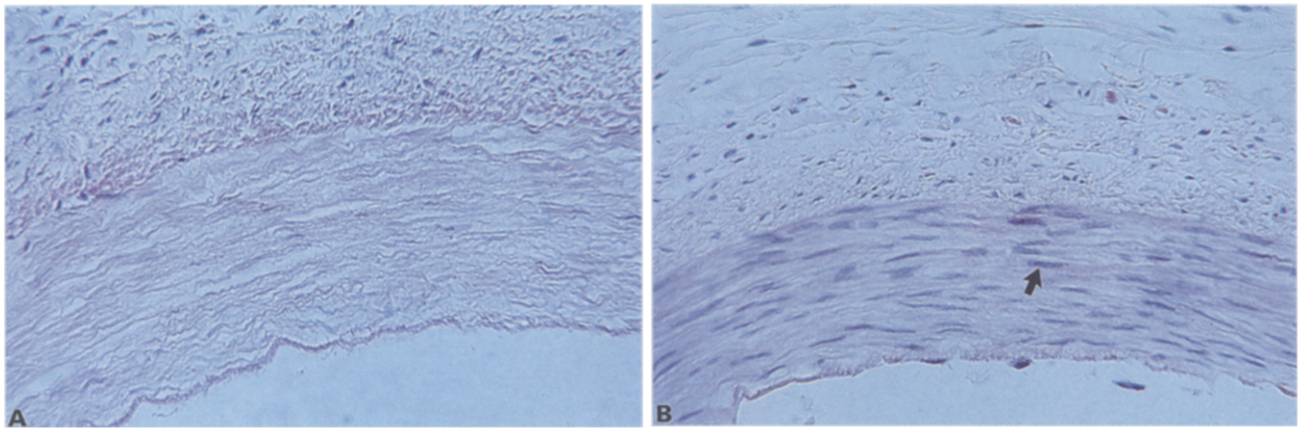

Fig. 4. Transverse haematoxylin and eosin sections (40 × objective, light microscopy) of a swine iliac artery (a) following treatment with PDT and (b) following a control laser alone irradiation. The arrow shows the presence of abundant VSMC nuclei in the control section with almost complete depletion in the PDT section.

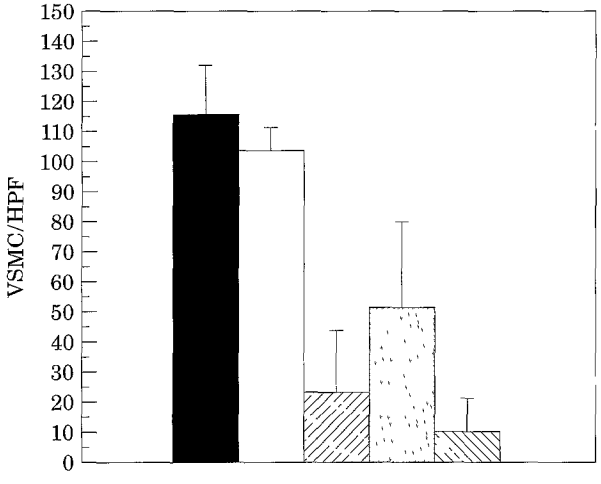

Fig. 5. Bar chart showing mean cell counts per HPF for control vessels and those exposed to PDT at 1 5, 2 5 and 6–7h following sensitisation from animals sacrificed at 3 days. Error bars represent standard deviations (■) ALA control, (□) laser control, (▨) PDT @ 1 5h, (▩) PDT @ 2 5h, (▧) PDT @ 6–7h

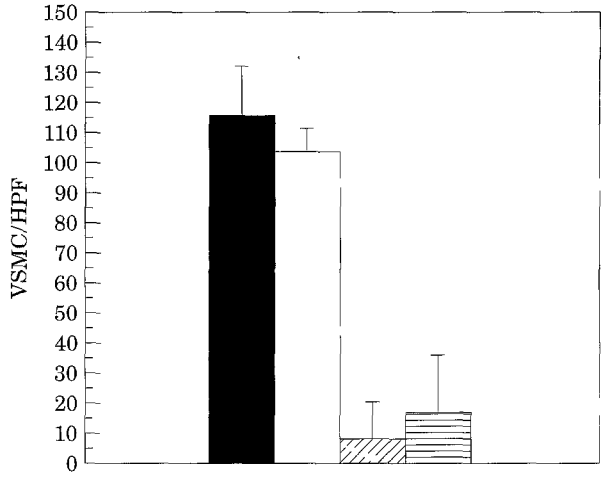

Fig. 6. Bar chart showing mean cell counts per HPF for control and treated vessels harvested at 3 and 14 days following PDT at the same drug light intervals. Error bars represent standard deviations (■) ALA control, (□) laser control, (▨) PDT-3 days, (▤) PTD-14 days

Discussion

This study has established the pharmacokinetics of 5-ALA in the pig model, shown that endovascular light delivery is feasible and that vascular PDT results in medial VSMC depletion. Endothelial cell regeneration occurred whilst VSMC depletion persisted and despite a period of endothelial denudation, no neointimal hyperplasia occurred. The pharmacokinetic studies showed that a peak in PpIX activity in the media occurred much later than that expected from small animal data. In Nyamekye's work[23] using 5-ALA in the rat carotid model, peak PpIX activity occurred at 1h post-dose in all layers, but differred in magnitude, the peak in the media being three times that of the intima or adventitia. The large animal data presented here, not only shows that the peak activity level within the media occurs between 5–8h, but also reveals that an equivalent peak occurs in the adventitia, but much earlier.

This has important implications, firstly for the timing of PDT to reduce intimal hyperplasia and secondly, the differential photosensitisation of the arterial wall raises the possibility that PDT could be used as an investigative tool for studying the role of individual wall layers in the restenosis process. It has already been suggested that the adventitia plays a more important role in restenosis than

previously thought,[28,29] but current techniques available for studying the adventitia in isolation are rudimentary.[30]

From the above pharmacokinetic studies, PDT was given at a number of time points to coincide with maximal sensitiser activity in the adventitia, then media, and finally when activity was at a minimum. The results of these experiments confirm the data from small animal studies, i.e. that PDT can exert an effect on the media which results in VSMC depletion. Treatments at 5–7h post-dose showed a significant reduction in the number of VSMCs within the media compared with controls. What is perhaps more surprising is that PDT given at 1.5h post sensitiser (coinciding with a peak in adventitial activity) produced a similar depletion in the medial VSMC population. An obvious explanation for such an effect would be that the outcome as measured was far too sensitive and that PDT at any time after photosensitisation would produce the same effect. If this was the case however, treating at 2–2.5h (with minimum activity in all layers) would lead to a comparable VSMC depletion. Although PDT at this time does show some depletion compared with controls, it is of the order of 50% rather than 90% depletion obtained at the other time points. The above variation in efficacy of PDT with time mirrors the fluorescence profile of 5-ALA, which lends validity to the observation that PDT at a time of selective adventitial sensitisation can cause medial call depopulation. This may be due to a direct effect by oxygen radicals generated in the adventitia causing medial cell death or may be indirect, with adventitial cell death impairing nutrition of the media.

For PDT to be a practical adjunctive treatment to prevent restenosis after PTA, it would have to be delivered at the time of PTA and via a percutaneous technique. Previous vascular PDT studies have relied on external irradiation of an open artery, although Nyamekye *et al.*[23] did experiment with an intra-arterial laser fibre. As well as satisfying the minimally invasive principles imperative in any percutaneous technique, intraluminal light delivery via a PTA balloon would solve two important problems; that of centring and excluding blood from the treatment field. Vincent[31] has previously established that blood significantly limited the transmission of red light, but the ability to irradiate via an inflated balloon effectively isolates a blood-free segment of arterial wall for treatment. At the same time, the radially diffusing laser fibre within the central channel of the balloon leads to a relatively uniform circumferential treatment area. The development of a transparent PTA balloon has facilitated this and also established that the intra-arterial delivery of PDT with 5-ALA as a sensitiser is feasible and effective. Moreover, use of equipment currently used in clinical practice will make a clinical trial of the technique a practical prospect with minimal modification. In the present study, up to 1950s were needed to produce 50 J/cm^2 over the 4cm length of the balloon, but in recent studies, optimisation of laser function has reduced this time to 500s.

No complications were seen in any of the treated or control pigs. In particular, neither acute thrombosis nor aneurysmal formation occurred. Although one may not expect the latter to have occurred in the time frame studied, there is evidence that the cellular depletion caused by PDT does not influence the mechanical integrity of the arterial wall. Grant[32] has shown that the bursting pressure of arteries following PDT treatment using 5-ALA is no different from that of controls. Thrombosis, if it was going to occur, would have been most likely in the first week, as there was denudation of the endothelial layer at 3 days. All pigs were given heparin intraoperatively and aspirin perioperatively and by 14 days, marker studies showed complete re-endothelialisation. It would therefore be unlikely that thrombosis would occur later than this.

Therapies directed purely at inhibiting neointimal hyperplasia have subsequently failed to prevent restenosis clinically and in large animal models. The emergence of remodelling as a concept was used as an explanation for the failure of such therapies to completely abolish restenosis. Stenting inhibits both immediate negative recoil and remodelling. This work does not address the influence of PDT on the arterial response to injury and remodelling, but does show that persistent VSMC depletion can be achieved whilst allowing re-endothelialisation. This may prove particularly useful as a therapy to reduce the incidence of in-stent restenosis which is almost exclusively secondary to intimal hyperplasia.[16] Currently there is much interest in ionising brachytherapy as a means of reducing restenosis[33–35] and although early clinical results look promising,[36] the known long-term effects of ionising irradiation may point to problems in the future.[37] It is possible that endovascular PDT using 5-ALA as a sensitiser is a safer but just as efficacious alternative.

In conclusion, this study confirms the efficacy of vascular PDT using 5-ALA as a photosensitiser in the pig model. It shows that endovascular delivery of light via an appropriate PTA balloon is practical and does

not give rise to thrombotic complications. We have since established the effectiveness of PDT in reducing intimal hyperplasia using the same technique in a balloon injury model and are now in a position to proceed with clinical trials.

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