Targeting Oxidative Stress after Percutaneous Coronary Intervention

by

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Watt J, Wadsworth RM, Kennedy S, Oldroyd KG. Pro-healing drug-eluting stents: a role for antioxidants? Clinical Science 2008;114:265-273.

Watt J, Wadsworth RM, Kennedy S, Oldroyd KG. The effect of reactive oxygen species on whole blood aggregation and the endothelial cell-platelet interaction in patients with coronary heart disease. Life Sciences 2007 (2007) Proc Life Sciences, PC15 (Abstract).

Watt J, Wadsworth RM, Kennedy S, Oldroyd KG. Reactive oxygen species inhibit whole blood aggregation in patients with coronary heart disease. Medical Research Society Communications 2007, February 28 (Abstract).

List of abbreviations

ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
ARB	Angiotensin receptor blocker
BMS	Bare metal stent
CABG	Coronary artery bypass graft
CART-1	Canadian Antioxidant Restenosis Trial-1
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CL	Chemiluminescence
DES	Drug-eluting stent
DPI	Diphenyleneiodonium
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EEL	External elastic lamina
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
EPR	Electron paramagnetic resonance
FGF	Fibroblast growth factor (?once)
H_2O_2	Hydrogen peroxide
HPLC	High-pressure liquid chromatography
IEL	Internal elastic lamina
IL	Interleukin
ISR	In-stent restenosis

IVUS	Intravascular ultrasound
KDR	Kinase insert domain receptor/VEGF receptor 2
LDL	Low-density lipoprotein
L-NAME	N_{ω}-nitro-L-arginine methyl ester
LV	Left ventricular
M	Moles per litre
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
NADPH	Nicotinamide adenine dinucleotide phosphate
NAOH	Sodium hydroxide
NIH	Neointimal hyperplasia
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NS	Non-significant
O_2^-	Superoxide anion
OH^-	Hydroxyl anion
$ONOO^-$	Peroxynitrite
OxLDL	Oxidised low-density lipoprotein
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PIS	Participant information sheet
PRP	Platelet-rich plasma
QCA	Quantitative coronary angiography

RES	Rapamycin-eluting stent
RLU/s	Relative light units per second
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of the mean
SES	Succinobucol-eluting stent
SIN-1	3-morpholinosydnonimine
SIRT	Sirtuin (silent mating type information regulation 2 homolog)
SMC	Smooth muscle cell
SOD	Superoxide dismutase
SRES	Succinobucol/rapamycin-eluting stent
tPA	Tissue plasminogen activator
UK	United Kingdom
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WBA	Whole blood aggregation
X	Xanthine
XO	Xanthine oxidase

Declaration

The design of the work contained in this thesis was provided by myself and my supervisors, Dr. Keith G. Oldroyd, Professor Roger M. Wadsworth and Dr. Simon Kennedy. I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not previously been submitted for a higher degree.

Dr. Jonathan Watt

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Summary

Percutaneous coronary intervention (PCI) improves the blood supply to the heart by unblocking narrowed coronary arteries. Implantation of a coronary stent is usually required to scaffold the artery and improve long-term vessel patency. Drug-eluting stents (DES) have been developed to decrease the incidence of stent renarrowing, known as in-stent restenosis (ISR), the main limitation of bare metal stents (BMS). DES release potent drugs into the artery wall to inhibit cell division and attenuate ISR. However, this strategy can also impair vascular healing and increase the risk of stent thrombosis, which is a serious concern. Novel approaches to this problem are urgently required. Oxidative stress reflects a state in which reactive oxygen species (ROS) prevail over antioxidant defences. PCI causes a major release of ROS from the injured artery wall and these molecules appear to play an important role in critical signalling pathways involved in vascular repair. Numerous animal studies have found that oral antioxidants may reduce ISR and improve healing, yet these strategies have not been effective in humans. Stent-based delivery of antioxidants may offer more efficacious, targeted protection against oxidative stress than oral administration. The role of oxidative stress in endothelial repair mediated by bone marrow-derived endothelial progenitor cells (EPCs) in patients with coronary heart disease is also poorly defined. The main aims of this thesis were: to determine the *in vitro* effects of oxidative stress on key aspects of thrombosis and vascular healing; to evaluate a novel antioxidant-eluting stent in an *in vivo* porcine model; and to examine the relationship between oxidised low-density lipoprotein (oxLDL), EPCs and coronary endothelial function in patients with stable angina.

Oxidative stress, generated by the xanthine/xanthine oxidase reaction, inhibited whole blood aggregation in a concentration-dependent fashion. This was probably due to an excess of ROS which impaired, rather than stimulated, thrombosis. Healthy endothelial cells (ECs) also inhibited whole blood aggregation, but this was not mitigated by oxidative stress. EC migration was assessed using an *in vitro* endothelial wound scratch assay. Oxidative stress was highly toxic to ECs and inhibited migratory activity. Nitrone D, a novel spin trapping antioxidant, was evaluated for its suitability as a novel DES coating. Nitrone D displayed weak antithrombotic effects, but markedly inhibited EC migration. Nitrone D was therefore unsuitable for a DES that was intended to improve re-endothelialisation.

Oral probucol has established efficacy in animal models of restenosis, but not in humans. Probucol has been successfully incorporated as a dual DES coating with rapamycin in clinical trials. Succinobucol is a novel derivative of probucol with more potent antioxidant, anti-inflammatory and antiproliferative effects. A novel polymer-free succinobucol-eluting stent (SES) and succinobucol/rapamycin-eluting stent (SRES) were developed and compared to a commercially available polymer-free rapamycin-eluting stent (RES) and BMS. Pharmacokinetic studies demonstrated optimal drug elution from the SES. However, in a porcine coronary model, the SES significantly increased neointimal thickness and aggravated ISR. The RES reduced neointimal thickness non-significantly, whereas the SRES caused no difference in neointimal thickness, compared with the BMS. The SES was associated with greater inflammation and persistent fibrin deposition around the stent struts, which are signs of defective healing. There were no significant

differences in endothelial regeneration between the groups. Subsequent cell culture studies found that succinobucol was toxic to ECs and smooth muscle cells. In the clinical study, circulating levels of EPCs were strongly correlated with coronary endothelial function, which is a novel finding. Plasma oxLDL levels were not correlated with EPCs or coronary endothelial function.

In conclusion, ROS reflect a large array of molecules released after PCI that are multi-faceted regulators of platelets and vascular cells. As such, they represent a complex target for novel DES technologies. Excessive ROS may inhibit thrombus formation and delay re-endothelialisation. However, potent antioxidants delivered to injured arterial tissue after PCI may not necessarily encourage the physiological processes required to accelerate vascular repair. At high dose, local delivery of antioxidants may actually promote inflammation and aggravate ISR. Although oxLDL is known to induce endothelial dysfunction, it is not correlated with the number of circulating EPCs. These findings underline the complicated role of oxidative stress in vascular repair after PCI. Further studies are required to clarify whether antioxidants will ever provide advantages over existing options in the rapidly evolving field of interventional cardiology.

Chapter 1

Introduction

1.1 Preamble

Coronary heart disease (CHD) is the leading cause of death and disability in the developed world. In 2007, the age-standardised mortality rates from CHD in Scotland were 89 per 100 000 men and 30 per 100 000 women, which are the highest in the United Kingdom (UK) (1). Although the incidence of CHD has decreased over the past decade, the economic burden is huge, costing the UK around £9.0 billion a year in 2006 (1). As a result, much effort is directed towards the development of effective, safe and lasting treatments for CHD.

Initial management of CHD includes lifestyle modification and drug therapy which aim to reduce myocardial ischaemia and prevent myocardial infarction. Despite these measures, many patients remain symptomatic and therefore mechanical methods of improving coronary artery blood flow have been developed (coronary revascularisation). The earliest attempts to achieve coronary revascularisation led to the introduction of coronary artery bypass graft surgery (CABG), in which autologous venous and arterial grafts are used to bypass coronary artery stenoses or occlusions. CABG requires a general anaesthetic and is a major open heart operation, with a modest but appreciable early post-operative risk of death (1-2%), a typical hospital stay of several days and a rehabilitation period of several months.

Percutaneous coronary intervention (PCI) offers an alternative and much less invasive method of coronary revascularisation. It is performed under a local anaesthetic and involves percutaneous access to the coronary arteries via a

catheter which usually enters the circulation via the radial or femoral artery. Placement of a thin guide wire into a diseased coronary artery allows the passage of specialised equipment which is used to dilate a stenosis by inflation of a balloon catheter and ideally, deployment of an endovascular stent. PCI is capable of reopening a narrowed or blocked coronary artery (or coronary artery bypass graft), restoring normal epicardial blood flow and more importantly, re-establishing normal myocardial perfusion. Patients undergoing PCI typically require minimal preparation, recover within hours and can often be discharged on the day of the procedure. Although procedural mortality associated with PCI is very low (0.1%), many patients suffer from progressive renarrowing or sudden blockage of the stent, therefore, the probability of requiring repeat revascularisation after PCI is currently higher than that after CABG (2). The natural history of PCI since inception has proven that refinement of technology can help to improve this situation, but current devices have brought with them their own limitations. Consequently, the discovery of novel approaches to improve the performance of stents remains crucial to the future development and success of PCI.

1.2 PCI

1.2.1 Historical aspects

In 1964, Dotter and Judkins were first to report a new percutaneous technique to treat lower limb arteriosclerotic disease (3). However, it wasn't until 1979 that Gruntzig *et al.* (4) published the first case series of 50 patients in whom percutaneously introduced inflatable balloon catheters were used to treat

coronary artery stenoses. The success of early balloon coronary angioplasty was tempered by the relatively high incidence of acute vessel closure and chronic renarrowing (restenosis), which often led to recurrent symptoms and a requirement for repeat procedures. In 1985, Palmaz *et al.* published the successful use of an endovascular metallic stent to prevent acute vessel recoil and limit late negative remodelling after balloon dilatation in dogs (5). The following year, Sigwart and Puel reported the first case series of patients who received self-expandable stainless steel stents to treat ilio-femoral and coronary artery disease after failed balloon angioplasty or CABG (6). Due to the major reduction in the rate of restenosis and requirement for repeat revascularisation, there was a massive expansion in the use of coronary stents worldwide. By the end of the 1990s, stents had become integrated into almost all PCI procedures. Like other modern medical treatments, PCI was subject to critical assessment of efficacy and cost-effectiveness and in 2000 the National Institute of Clinical Evidence published a report supporting the routine use of stents during PCI (7). The British Cardiovascular Intervention Society have reported national audit data showing that uptake of PCI to treat CHD has increased exponentially over the past twenty years, with 83 130 PCI procedures (1 345 PCI per million) performed in 105 centres in the UK in 2009, producing a ratio of PCI to CABG in excess of 3:1. (Figure 1.1) (8).

1.2.2 Clinical evidence

In patients with stable angina, PCI is effective mainly to reduce symptoms. A systematic review of 6 prospective randomised controlled trials of PCI versus

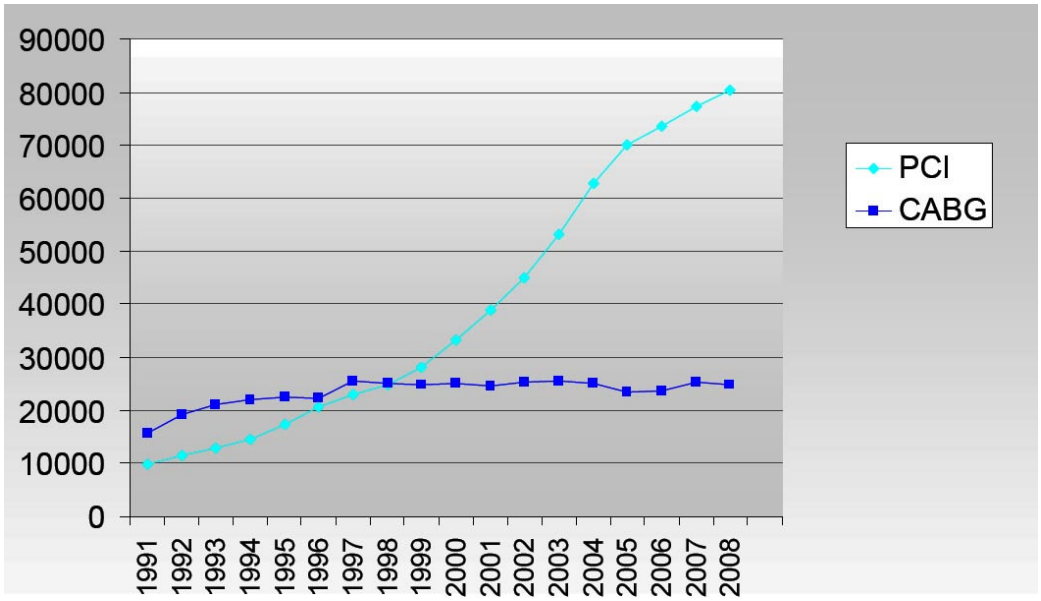

Figure 1.1 PCI versus CABG frequency in the UK.

An exponential rise in PCI procedures has occurred since the early 1990s, whereas the number of CABG operations has remained fairly constant.

medical therapy in stable angina confirmed an improvement in symptoms and quality of life, but no significant differences in the subsequent risk of death or myocardial infarction (9). The majority of trials included in this review were performed in the 1990s, when most patients received balloon angioplasty only. The routine use of stents has considerably reduced the requirement for repeat revascularisation compared to balloon angioplasty, however a major randomised controlled trial in 2 287 stable angina patients failed to demonstrate a reduction in death or myocardial infarction, compared to optimal medical therapy (10). This trial was limited by the exclusion of high risk patients and crossover to revascularisation in around 30% of the medical therapy group during the course of the study. A large meta-analysis comparing PCI versus CABG in stable angina has yielded no difference in mortality or myocardial infarction, especially when stents are utilised (11).

PCI can also be used to treat acute coronary syndromes, such as unstable angina or acute myocardial infarction. After non-ST elevation myocardial infarction (NSTEMI), an early invasive revascularisation strategy incorporating PCI or, to a lesser extent CABG, has been shown to prevent subsequent myocardial infarction and recurrent angina (12). Five year follow up data from a large UK randomised clinical trial has shown that high risk patients with non-ST elevation myocardial infarction achieve an overall mortality benefit with the routine use of invasive management (13). PCI for the treatment of ST elevation myocardial infarction (STEMI) has clear benefits compared with drug therapy and is now the preferred reperfusion strategy in the UK. A systematic review of the evidence for PCI to treat STEMI has demonstrated reductions in death, myocardial infarction and stroke, compared with thrombolytic drugs (14).

1.2.3 Coronary stents

The introduction of coronary stents to improve the performance of balloon dilation alone has been a major advance. The basic form of an uncoated coronary stent is known as a bare metal stent (BMS). The stent must be biologically inert and ideally provide a non-thrombogenic surface for blood flow. It must possess good radial strength to scaffold the artery reliably; however it must also be conformable to tortuous coronary anatomy and vessel irregularities. The field of stent design is continually evolving, although most BMS consist of a stainless steel or cobalt chromium tube into which laser guided cuts are made. The unexpanded stent is pre-mounted onto an angioplasty balloon catheter and is visible fluoroscopically during transit through the coronary circulation. When the

stent is optimally positioned over a coronary stenosis, the stent is deployed by inflating the balloon to high pressure (e.g. 14 atmospheres). The balloon is then fully deflated by applying negative pressure, which leaves the stent fully expanded against the artery wall before the balloon catheter can be withdrawn.

Stents compress the fissured intima against the underlying media, reducing the propagation of arterial dissection after balloon dilation. Moreover, by scaffolding the artery, the lumen is protected from acute elastic recoil and chronic negative remodelling of the artery, which are the main causes of restenosis following balloon angioplasty (15). Due to their superior efficacy compared with balloon angioplasty alone, stents are now utilised in over 90% of PCI procedures (8). However late luminal loss is not abolished by stenting. Renarrowing still occurs and is caused primarily by excessive growth of neointimal tissue as a response to the deep vessel wall injury resulting from stent struts and the presence of a foreign body. This phenomenon is known as in-stent restenosis (ISR), which occurs to a greater extent than after balloon angioplasty and an example is shown in Figure 1.2.

1.2.4 In-stent restenosis

ISR occurs in 10-50% cases, depending on various stent, lesion and patient characteristics (16). The main predictors of ISR are the presence of diabetes mellitus, the treatment of long coronary lesions and small vessel diameter. ISR usually presents as a recurrence of stable angina symptoms but in around 10% of cases it can present as an acute coronary syndrome (17).

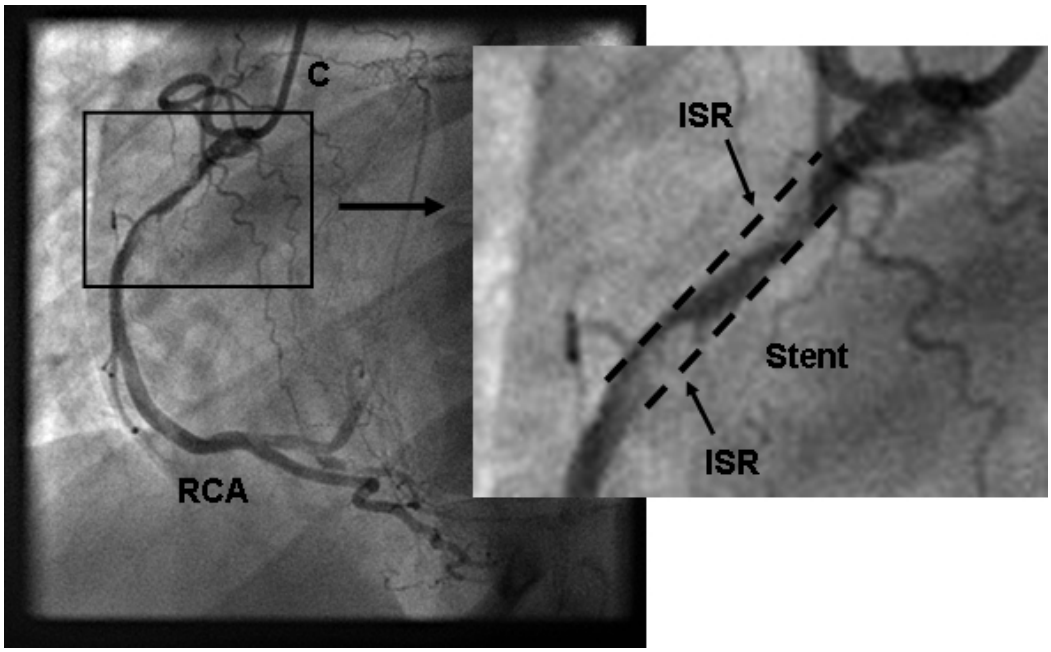

Figure 1.2 Angiographic illustration of ISR.

An angiogram of the right coronary artery (RCA) shows a coronary catheter (C) placed at the ostium of the RCA, following injection of radio-opaque contrast. The magnified insert on the right demonstrates a previously implanted stent in the proximal segment of the artery (position of struts indicated by broken parallel lines) which has developed partial obstruction. This is due to encroachment of the lumen by neointimal tissue, causing ISR (visualised as a loss of contrast density within stent).

The process of ISR resembles the natural wound healing process following vessel injury, typically occurring over several weeks to months. An integrated view of the molecular and cellular events leading to ISR has been proposed by Welt and Rogers (18). Stent deployment causes severe endothelial denudation, which leads to platelet and fibrin deposition on the injured artery wall. Activated platelets expressing adhesion molecules, such as P-selectin, attach to circulating leucocytes via platelet receptors, such as P-selectin glycoprotein ligand. Leucocytes roll along the injured wall and then bind tightly to the surface through the leucocyte integrin Mac-1 (CD11b/CD18) class of adhesion molecules via direct attachment to the platelet receptor glycoprotein 1bα (GP1bα) and through cross-linking with fibrinogen to the GPIIbIIIa receptor. Under the influence of chemokines, such as monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and IL-8, released from vascular smooth muscle cells (SMCs) and resident macrophages, leucocytes infiltrate the artery wall. Growth factors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) are released from platelets, leucocytes and SMCs, which stimulate SMCs to proliferate and migrate from the media into the neointima. Ongoing SMC proliferation, macrophage infiltration and extracellular matrix production cause neointimal hyperplasia (NIH); the pathognomic feature of ISR. Although the luminal renarrowing due to elastic recoil and negative remodelling common after balloon angioplasty is prevented by the stent struts, the neointimal response is exaggerated and proportional to the degree of stent injury (19). Endothelial repair involves endothelial cell (EC) migration, which may be the critical and rate-limiting

initiating step, followed by EC proliferation (20). As the denuded endothelium regenerates, it completely recovers the neointimal surface, allowing the vessel to resume normal function.

1.2.5 Endothelial regeneration

The vascular endothelium lines the circulatory system throughout the body and provides a highly selective permeability barrier to contain the blood within the vascular space. The endothelium is integral to the control of several key vascular functions and provides extensive signalling capabilities to other cells via the release of cytokines and growth factors. Delayed or incomplete healing of the endothelium after PCI is undesirable for a number of reasons.

First, the early regrowth of a functional endothelial layer is capable of attenuating NIH, the principle cause of ISR. After injury, ECs regulate SMC proliferation by the release of well-characterised growth promoters and inhibitors (21). Damage to the endothelium results in the activation of SMC proliferation, whereas repair of the endothelium leads to the reversal of this process and restoration of SMC quiescence (22). Endothelial progenitor cells (EPCs), derived from the bone marrow, also appear to contribute to endothelial regrowth, and intravenous transfusion of EPCs has been shown to reduce NIH in a mouse model of arterial injury (23). An early pilot study in patients using stents coated with anti-CD34 antibodies to target EPC surface antigens (Genous™ Bio-engineered R stent, OrbusNeich, Hong Kong) demonstrated clinical efficacy with a low incidence of ISR (24). However, more recently the Genous™ stent was associated with a poor performance in lesions at higher risk of ISR, with

antirestenotic efficacy approaching that of a BMS, albeit with no cases of stent thrombosis (25). Gene transfer of VEGF in a rabbit model of balloon injury has inhibited NIH in some cases (26), but not in others (27), and the role of catheter-based local VEGF gene delivery after human PCI is under investigation (28). A strategy utilising VEGF gene-eluting stents may be effective if the results of a promising rabbit study can be reproduced (29), although one clinical study had disappointing results (30).

Second, the regeneration of the endothelium is crucial to restore normal vascular tone through the action of a number of vasoactive substances, including nitric oxide (NO) and prostacyclin. Coronary endothelial dysfunction is characterised by a reduction in NO bioavailability and is a recognised precursor to the development of coronary artery disease and subsequent vascular events (31). Therefore, it is conceivable that persistent endothelial dysfunction after PCI would increase the risk of late procedural complications.

Finally, and particularly after stent deployment, regrowth of a functional endothelium is fundamental to luminal patency. An intact endothelial monolayer provides a non-thrombogenic surface for blood flow and acts as the primary regulator of haemostasis and thrombosis. The endothelium also serves as a non-permeable barrier between circulating platelets and agonists, such as collagen, widely present in coronary plaque and artery wall, which profoundly stimulate platelet aggregation. The endothelium restricts exposure of blood coagulation factors to the subendothelial layer, which is rich in tissue factor, the primary trigger of the coagulation system. ECs themselves directly inhibit thrombosis via

the antiplatelet effects of released NO (32), prostacyclin (33) and CD39, an ecto-ADPase (34). Endothelial-derived heparan sulfate, thrombomodulin and tissue factor pathway inhibitor are inhibitors of coagulation (35). The endothelium also regulates the endogenous fibrinolytic system by the release of endothelial tissue plasminogen activator (tPA), which plays a significant role in thrombus dissolution during acute coronary syndromes (36). Thus, in the milieu of an intrinsically thrombogenic metal stent, an absent or functionally deficient endothelium significantly impairs the natural defence of the stented coronary artery against acute occlusion, an event known as stent thrombosis.

1.2.6 Stent thrombosis

Implantation of a coronary stent immediately creates an intensely prothrombotic environment, which can lead rapidly to stent thrombosis (figure 1.3). As well as denuding the endothelium and exposing the intimal contents, the stent itself is highly thrombogenic. Platelet activation and aggregation are unavoidable consequences of arterial injury and preventative measures must be employed to prevent stent thrombosis. Improved operator technique and newer equipment have reduced the incidence of acute stent thrombosis, but the greatest impact has been made by the adoption of intensive adjunctive antithrombotic regimens. This usually includes at least two different antiplatelet drugs (aspirin, clopidogrel and, in high risk cases, a glycoprotein 2B/3A inhibitor) and intravenous anticoagulant therapy (heparin or bivalirudin). Despite this, stent thrombosis remains one of the most serious and life-threatening complications of PCI. Stent thrombosis is often a catastrophic clinical event, resulting in a large myocardial infarction with a high risk of death (37).

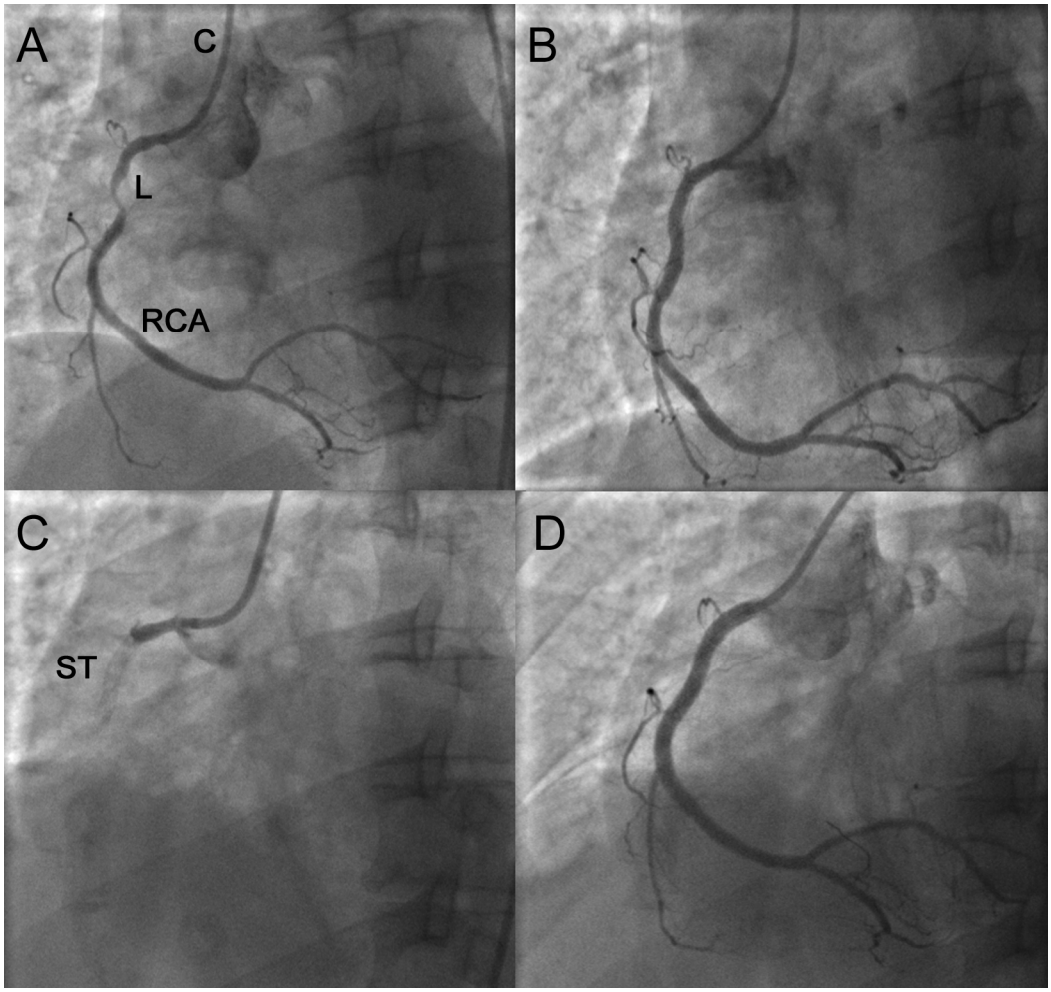

Figure 1.3 Angiographic illustration of stent thrombosis.

A, Coronary angiogram with a coronary catheter (C) placed at the ostium of the right coronary artery (RCA) with a stenotic lesion (L) in the proximal segment. B, Angiographic result following dilation of the lesion and coronary stent implantation. C, Repeat angiogram several days later shows an early stent thrombosis (ST) with occlusive thrombus blocking the stent, which caused a large inferior myocardial infarction. D, Final angiographic result after the stent thrombosis has been treated using thrombus aspiration and further stenting.

Overall, the risk of stent thrombosis is related to a large number of patient, procedural and stent factors (Figure 1.4). Premature discontinuation of antiplatelet drug therapy is the strongest predictor of stent thrombosis and patients with renal failure, diabetes or acute coronary syndrome are also at higher risk (38;39). Patients undergoing non-cardiac surgery after coronary stenting are at greatly increased risk of stent thrombosis and associated cardiovascular complications during the first two months after PCI (40) and therefore non-cardiac surgery should be deferred for several months unless absolutely necessary. A recent study showed no difference in the rates of stent thrombosis between BMS and drug-eluting stents (DES) in the early post-operative period, although important data on antiplatelet prescribing in either group were not reported (41). It is now recommended that dual antiplatelet therapy is continued where possible during the perioperative period to mitigate against the risk of stent thrombosis, unless there is an unacceptable risk of serious bleeding (42), such as neurosurgery.

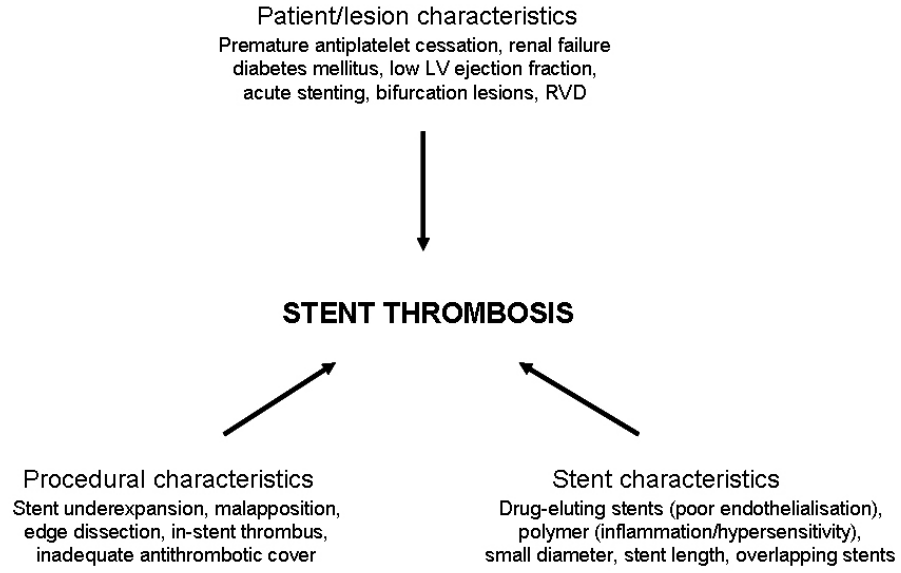

Figure 1.4 Risk factors for stent thrombosis.

LV, left ventricular; RVD, reference vessel diameter.

In randomised clinical trials, stent thrombosis typically occurred after approximately 1-2% of PCIs (43;44). However, observational data from patient registries suggests the incidence of stent thrombosis in real world populations may be higher. Combined data from two large European centres (including 8 146 patients) reported a 3 year incidence of angiographically proven stent thrombosis of 3%, occurring at a constant rate of 0.6% per year after the first year. (39). A registry of 5 842 ST-elevation myocardial infarction patients reported a 3.5% incidence of angiographically confirmed stent thrombosis within a month of primary PCI, of which half occurred during the first 24 hours (45). This discrepancy underlines the differences in patient populations selected for randomised clinical trials compared with contemporary clinical practice, with the latter group having more severe underlying disease, greater comorbidity and more complex PCI, increasing the risk of stent thrombosis (39).

In order to more accurately quantify the incidence of stent thrombosis across different populations and make comparison between studies, the Academic Research Consortium (ARC) have introduced a classification system which has standardised the reporting of such events (46), the true incidence of which may be underestimated if based solely on angiographic confirmation (43). Definite stent thrombosis requires the presence of an acute coronary syndrome with angiographic or post-mortem evidence of thrombus or occlusion. Probable stent thrombosis includes unexplained death within 30 days of the procedure or acute myocardial infarction involving the vessel territory of the implanted stent without angiographic confirmation. Possible stent thrombosis includes all unexplained deaths occurring at least 30 days after the procedure. Using this classification

system, the cumulative rate of definite and probable stent thrombosis probably more accurately reflects and is now commonly used to approximate the incidence of stent thrombosis in research studies.

Stent thrombosis is also categorised as early, occurring between 0-30 days (this can be sub-classified further into acute within 24 hours and subacute after 24 hours); late, occurring between 31 days and 1 year; or very late, occurring more than 1 year after stent implantation (46). This distinction is important because stent thrombosis may occur at different time periods after PCI due to different pathophysiological processes.

The first month after PCI represents the period when patients are at greatest risk of stent thrombosis (39). Early stent thrombosis is often associated with a procedural or technical problem with the stent. In an intravascular ultrasound (IVUS) registry of 53 patients with definite early stent thrombosis, 94% of cases demonstrated at least one abnormality on the original IVUS at the time of index PCI, such as undersizing of the stent, strut malapposition to the surrounding artery wall, edge dissection, thrombus formation or untreated disease at the inflow or outflow of stent (47). A registry of 201 early stent thromboses after ST-elevation myocardial infarction discovered that unrecognised dissection, stent undersizing and small stent diameter were the strongest predictors for acute stent thrombosis (45). Lack of dual antiplatelet therapy or non-cardiac surgery are major risk factors for subacute stent thrombosis (38;41;45). The distinction between BMS and DES does not appear to play an important role in early stent thrombosis, as even BMS require at least one month to re-endothelialise and

therefore are both at increased risk of stent thrombosis during this time period, particularly if other risk factors are present. Late stent thrombosis occurs less frequently than early stent thrombosis although early discontinuation of antiplatelet therapy remains an important trigger (38;48). More complex PCI such as bifurcation stenting is also associated with higher risk during this period (38). Very late stent thrombosis, occurring at least one year after stenting, usually reflects impaired endothelial healing or persistent drug/polymer toxicity. The risk of stent thrombosis for BMS during this period is relatively low, because the stent struts are generally covered with new endothelial and neointimal tissue. The limitations of DES with regard to suboptimal healing and their increased risk of very late stent thrombosis will be discussed in more detail in section 1.2.7. A Japanese registry of 294 212 PCI procedures (exclusively sirolimus-eluting stents) yielding 611 ARC-definite stent thromboses confirmed previous studies that the majority of events occur within the first month after stenting (49). Characteristic factors predicting stent thrombosis after one month (late or very late stent thromboses) versus early stent thrombosis included haemodialysis, end-stage renal disease, chronic total occlusions and age <65 years. Exploratory analyses demonstrated some differences in patient characteristics between late and very late stent thrombosis groups, with older age, lower body mass index and greater burden of vascular disease or diabetes more prevalent in patients with late as opposed to very late events, suggesting that comorbidity is a more prominent factor during the late period whilst usually receiving dual antiplatelet therapy. The one year mortality rate after late stent thrombosis appears to be higher than after acute or very late events (45;49), probably due to differences in presentation and important clinical factors as described above.

1.2.7 Drug-eluting stents

Implantation of a metal stent into a diseased coronary artery has provided the cardiologist with a unique opportunity to deliver drugs directly into the surrounding artery wall in an attempt to modify the maladaptive thrombotic and hyperplastic responses to vessel injury and improve clinical performance. Local drug delivery from a DES aims to reduce the toxicity and compliance issues related to systemic administration. In order to control the drug release from the stent platform, the drug is eluted in a pre-specified pattern. To achieve this, the active drug is commonly carried by a polymer. The ideal polymer is biologically inert and should not promote inflammation or hypersensitivity because this may, in itself, lead to ISR or stent thrombosis (50).

Clinical efficacy of first generation DES

The first DES introduced to clinical practice was the sirolimus-eluting stent. Sirolimus (also known as rapamycin) is a natural macrocyclic lactone, which is a product of the bacterium *Streptomyces hygroscopicus*. Sirolimus acts by inhibiting the mammalian target of rapamycin (mTOR). It has significant anti-inflammatory and immunosuppressive effects and is used to prevent rejection after organ transplantation. Recognition of its potent antiproliferative properties led to clinical studies, which confirmed its efficacy as an antirestenotic stent coating. The Cypher® stent (Cordis, Johnson and Johnson) is a commercially available sirolimus-eluting stent and utilises a stainless steel stent and permanent polymer to control drug release. Early human experience with both slow and fast release profiles for the sirolimus-eluting stent was favourable, demonstrating long term efficacy in the prevention of NIH, particularly with the slow release

version which is currently used in clinical practice (51). There have been many large randomised controlled trials comparing sirolimus-eluting stents with BMS in stable CHD. Early trials such as RAVEL (52) and SIRIUS (53) were first to report. In a subsequent meta-analysis, it was found that sirolimus-eluting stents led to an impressive reduction in ISR in the first year compared with BMS, from 37% to 6% respectively (54). In addition, large improvements in target lesion revascularisation were found comparing sirolimus-eluting stents with BMS, from 19% to 4% respectively. Up to a year, there was no difference in the incidence of myocardial infarction or death in patients with the sirolimus-eluting stent. It was worth noting that these trials involved relatively low risk patients and lesions, often in the absence of multiple medical comorbidities or presence of multivessel disease, which is misrepresentative of contemporary interventional practice. The real world effectiveness of sirolimus-eluting stents has been examined in prospective registries, and these have shown repeat revascularisation rates of up to 10% within a year in more complex cases (55).

Paclitaxel-eluting stents were next to show encouraging results. Paclitaxel is an anticancer drug which also has potent antiproliferative effects. Paclitaxel-eluting stents are currently marketed as the Taxus® stent (Boston Scientific). The main source of evidence came from the TAXUS trial program (56). Paclitaxel is also carried in a permanent polymer, and both slow and moderate release profiles have been studied. Although both DES provided excellent suppression of NIH, the slow release Taxus® is currently the only commercially available version. A meta-analysis of the TAXUS program demonstrated a reduction in ISR using the

Taxus® from 24% to 7%, and reductions in target lesion revascularisation from 12% to 3%, compared with BMS (54).

A comprehensive meta-analysis of all randomised trials and observational studies of first generation DES (sirolimus- or paclitaxel-eluting stents) in which greater than 100 patients were enrolled with at least one year follow-up was performed by Kirtane *et al*. (57). This demonstrated that in randomised trials (9 470 patients in 22 studies), first generation DES led to a significant reduction in target vessel revascularisation compared with BMS (hazard ratio, 0.45; 95% CI, 0.37 to 0.54), with no significant differences in mortality or myocardial infarction. When all observational studies were analysed (182 901 patients in 34 studies), DES were associated with a similar reduction in target vessel revascularisation (hazard ratio, 0.54; 95% CI, 0.48 to 0.61), but also a significant reduction in mortality (HR, 0.78; 95% CI 0.71 to 0.86) and myocardial infarction (HR, 0.87; 95% CI, 0.78 to 0.97), compared with BMS. Whilst accepting the potential for selection bias and residual confounding of observational data, this suggests that real-world use of DES retains efficacy outside of the carefully selected populations of randomised controlled trials and may be responsible for a lower rate of adverse cardiac events in higher risk patients with more complex CHD, compared with BMS. In the UK, DES are used in greater than 60% of PCI procedures, especially in diabetic patients and those patients with long or narrow coronary lesions, where the risk of ISR is higher and efficacy of DES greater in absolute terms (8).

Limitations of the first generation DES

First generation DES limit ISR by inhibiting the cell cycle of proliferating cells. Sirolimus-eluting stents also reduce MCP-1 and IL-6 expression (58), and paclitaxel is known to influence inflammatory cell adhesion (59). However, whilst they are designed to arrest vascular SMC growth, it is not surprising that these potent antiproliferative compounds also have long-term effects on other nearby cells. Importantly, paclitaxel inhibits EC migration, whereas both paclitaxel and sirolimus inhibit EC proliferation (60). Sirolimus also inhibits proliferation of EPCs (61). These toxic effects are evident in pathological examinations of sirolimus- and paclitaxel-eluting stents, which frequently show evidence of poor healing, characterised by persistent fibrin deposition and incomplete re-endothelialisation (62). In addition, chronic eosinophilic infiltration (hypersensitivity), probably due to the polymer, may be seen and these findings have been closely linked to stent thrombosis. As well as impairing the endothelium regrowth, both sirolimus- and paclitaxel-eluting stents induce endothelial tissue factor expression which promotes a thrombogenic environment (63). Angioscopic examination of the sirolimus-eluting stent *in situ* has visualised incomplete healing and exposed stent struts persisting at a time when a BMS has fully re-endothelialized, and this may be associated with subclinical thrombus formation (64-66). Sirolimus- and paclitaxel-eluting stents may also lead to an increased risk of late acquired malapposition (67); a finding which may predict stent thrombosis (47). The sirolimus-eluting stent has been shown to cause endothelial vasomotor dysfunction in the adjacent vessel segments (68), indicating functional impairment resulting from DES implantation.

Owing to suboptimal endothelial regeneration after DES treatment, combined antiplatelet therapy with aspirin and clopidogrel is extended by at least several months during the perceived re-endothelialisation period. If prolonged dual antiplatelet regimes are used, DES do not appear to increase the risk of stent thrombosis at 12 months compared with BMS (69). However, as follow-up continues beyond a year, there is concern that first generation DES may present an increased risk of late myocardial infarction and death, a clinical surrogate of stent thrombosis (70;71). Although complete patient level meta-analyses of the randomised trials with first generation DES demonstrated no overall difference in cardiovascular events compared with BMS, there is an increased risk of late stent thrombosis (72). Therefore, there is now a considerable need to ensure the next generation of DES are effective and safe, and that means ensuring the endothelium is able to regenerate in a timely fashion.

Recent developments in DES and polymers

Following the introduction of the sirolimus- and paclitaxel-eluting stents and especially since the reporting of late stent thrombosis, there has been interest in testing novel DES with alternative biological profiles. By modifying the chemical structure of sirolimus (rapamycin) slightly, it is possible to create derivatives with improved tissue absorption and differential effects on cell proliferation. Zotarolimus is a highly lipophilic sirolimus analogue with antiproliferative and anti-inflammatory properties. A zotarolimus-eluting stent has been tested as part of the Endeavor® stent program (Medtronic Vascular), utilising a cobalt alloy stent platform and biocompatible phosphorylcholine polymer. The zotarolimus-eluting stent effectively reduced ISR compared with

BMS (73), but led to higher rates of ISR and repeat revascularisation compared with first generation sirolimus-eluting stents (74). The thin-strut permanent fluoropolymer-based everolimus-eluting cobalt chromium stent (Xience V®; Abbott Advanced Cardiovascular Systems) has excellent efficacy in terms of reducing the incidence of ISR compared with BMS (75) and paclitaxel-eluting stents (76). 3 year randomised clinical trial data involving 1 302 patients in the SPIRIT II and SPIRIT III randomised trials reported a significant reduction in myocardial infarction and target lesion revascularisation in the everolimus-eluting Xience V® stent group compared with the paclitaxel-eluting Taxus® stent group. The cumulate rates of ARC-defined definite or probable stent thromboses were 1.2% in the everolimus-eluting stent patients versus 1.9% in paclitaxel-eluting stent patients, which was not significant (77).

Polymers used to coat the stent and act as a drug delivery mechanism for biologically active agents vary in suitability, with some causing a considerable increase in neointima formation in animal models (78). Although phosphorylcholine (which occurs naturally in red blood cell membranes) is thought to be a highly biocompatible polymer, its clinical use in DES (specifically zotarolimus-eluting Endeavor® stents) has been associated with increased rates of ISR compared with other durable polymeric DES platforms (73;74), most likely due to excessively rapid drug release. Furthermore, similar neointimal responses to BMS have been reported in some cases (79). A preclinical comparison of a phosphorylcholine versus a fluorinated copolymer (as found on everolimus-eluting Xience V® stents) in rabbit iliac arteries has

reported that the extent of re-endothelialisation and inflammatory response was similar (80).

Localized hypersensitivity reactions in the artery wall associated with long-term exposure to first generation DES polymers (62) have prompted attempts to provide effective drug elution without the use of a permanent polymer. One such development is the introduction of the bioabsorbable polymer, which degrades over 6-9 months, removing the chronic stimulus for polymer-induced inflammatory reactions after drug elution is complete. An everolimus-eluting stent with a bioabsorbable polymer (polylactic acid, which degrades into carbon dioxide and water) has undergone investigation, and early trials demonstrate a reduction in the incidence of ISR compared with BMS (81;82). Excellent efficacy and safety data have also been reported in the LEADERS randomised trial of a biolimus-eluting stent (Biomatrix™; Biosensors International) which also utilises a biodegradable polylactic acid polymer (83) and showed non-inferior efficacy to the polymeric sirolimus-eluting Cypher® stent. Although an optical coherence tomography substudy of the LEADERS trial demonstrated improved strut coverage in the Biomatrix™ compared with the Cypher® stent (84), a clear reduction in the risk of stent thrombosis using biodegradable stent polymers has not yet been shown.

Polymer-free DES technology

The ideal DES situation may be the carriage and controlled release of a drug from a stent without the necessity of a polymer, thereby obviating all potential toxicity. It has been shown in rabbit iliac artery stenting that whilst omission of a

polymer altogether may attenuate the neointimal suppression achieved by a DES (likely due to accelerated drug release), it may allow improved re-endothelialisation and reduced local arterial inflammation (85). Reasonable results were demonstrated by a non-polymer paclitaxel-eluting stent (86;87). If non-polymer DES can achieve improved control of drug elution, equivalent to polymeric DES, then improved efficacy would be expected. The Yukon® (Translumina®, Germany) stent platform incorporates a microporous surface to improve drug release characteristics and has been tested extensively as part of the ISAR-TEST clinical trial program. An early randomised clinical study by Dibra *et al.* initially showed that the surface modification of the Yukon® stent alone caused a trend to reduced ISR compared with smooth surface uncoated stents, suggesting that the microporous stent surface was highly biocompatible and suitable for DES investigation (88). A polymer-free rapamycin-eluting Yukon® stent was subsequently tested in the randomised controlled ISAR-TEST study in 450 patients and found to have equivalent efficacy to the permanent polymer paclitaxel-eluting Taxus® stent (89). Later studies compared the polymer-free Yukon® rapamycin-eluting stent with more effective DES platforms. Two year follow-up data from the ISAR-TEST-3 clinical study has shown that whilst that the polymer-free Yukon® rapamycin-eluting stent was associated with greater late loss in the first year, its efficacy was equivalent to both permanent polymer (Cypher®) or biodegradable polymer rapamycin-eluting stents after two years, due to late “catch-up” luminal loss in the polymer-based platforms (90). Further modification of the polymer-free Yukon® rapamycin-eluting stent has been investigated, leading to the incorporation of the antioxidant probucol as a dual stent coating. The addition of probucol has been shown to retard rapamycin

release analogous to that of a permanent polymer (91). A polymer-free Yukon® dual rapamycin/probucol-eluting stent (dual-DES) has delivered excellent clinical results as part of the ISAR-TEST-2 randomised clinical trial. This study in 1 007 patients found that the dual-DES led to significantly lower rates of ISR and target lesion revascularisation than the Endeavor® zotarolimus-eluting stent and a trend to lower rates compared with the Cypher® sirolimus-eluting stents after two years follow-up (92). Exploratory analysis of this study showed that the extent of late clinical restenosis (target lesion revascularisation between 1 and 2 years) was significantly greater in the Cypher® group, compared with the polymer-free dual-DES. Similar to previous trials, there was no significant difference in death, myocardial infarction or stent thrombosis, although these studies are not individually powered to detect differences in these outcomes.

Perhaps the final frontier for stent-based drug delivery is the fully bioabsorbable DES, so long as the mechanical properties of metal stents can be successfully reproduced (93). A landmark clinical study of a fully bioabsorbable everolimus-eluting stent utilising a polylactic acid polymer reported promising results, although the mean in-stent late loss of 0.48 mm and diameter stenosis of 27% after two years is inferior to contemporary DES (94). Future refinement of the bioresorbable scaffold is likely to allow these devices to compete with the next generation of metal stent based devices.

To summarise the DES story so far, the uptake of evolving technology has led to major improvements allowing the provision of lasting percutaneous coronary revascularisation for patients with CHD. Improvements in stent design and

incorporation of novel drugs and polymers with improved efficacy and biocompatibility have resulted in rates of ISR much lower to that first witnessed using BMS. In terms of antiproliferative drugs, limited preclinical data suggests that everolimus (95;96), tacrolimus (97;98) or biolimus (99) may have superior effects on EC biology compared with sirolimus. Increasing experience with biodegradable and polymer-free DES has hinted that the risk of late "catch up" restenosis and very late stent thrombosis (both occurring after one year) may be lower with these platforms. However, there is currently no consistent data yet to prove that either second or third generation DES result in lower rates of hard clinical events such as stent thrombosis, myocardial infarction and death. Evaluation of aggregate long-term efficacy and safety data from large-scale randomised trials of these newer DES is awaited with interest.

1.3 Oxidative stress

1.3.1 Oxidative stress and cardiovascular disease

Reactive oxygen species (ROS) are highly reactive molecules released from the artery wall and circulating cells that have a profound impact on many biological processes. Increased production of ROS is a characteristic feature of several cardiovascular diseases such as atherosclerosis, hypertension and diabetes mellitus. ROS are capable of oxidising other substances by becoming reduced themselves. Oxidative stress is a phenomenon which describes the net influence of oxidants such as ROS over antioxidant defences. Oxygen is an abundant molecule found in humans and the relatively stable oxygen molecule can undergo univalent reduction to form the superoxide anion (O_2^-), a powerful ROS

and a major component of oxidative stress. The formation of O_2^- occurs through the action of a number of enzymes, the foremost of which is believed to be nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) oxidase. This enzyme is active in ECs, vascular SMCs, leucocytes and platelets, often as a response to vascular injury.

Other enzymes contributing to oxidative stress include xanthine oxidase (XO), which generates O_2^- by catalyzing hypoxanthine and xanthine to uric acid, nitric oxide synthase (NOS), which generates the free radical NO from L-arginine and the lipoxygenases, which oxidise polyunsaturated fatty acids to hydroperoxy fatty-acid derivatives. Oxygen can also be converted into O_2^- nonenzymatically as a result of electron transfers in the mitochondrial electron transfer chain (100). O_2^- can be dismutated to form another biologically active ROS, hydrogen peroxide (H_2O_2) through the action of superoxide dismutase (SOD). H_2O_2 can be decomposed into water and oxygen by the enzyme catalase. Other highly influential ROS include peroxynitrite ($ONOO^-$), which is formed by the reaction between O_2^- and NO, and the hydroxyl anion (OH^-), which is generated by the reaction between O_2^- and H_2O_2. Both $ONOO^-$ and OH^- anions are very strong oxidants.

Oxidative stress is critically involved in the pathophysiology of atherosclerosis (Figure 1.5) (101). Circulating low density lipoprotein (LDL) in the blood is oxidised by ROS to form oxidised low-density lipoprotein (oxLDL). OxLDL is capable of causing damage to the vascular endothelium and this promotes the

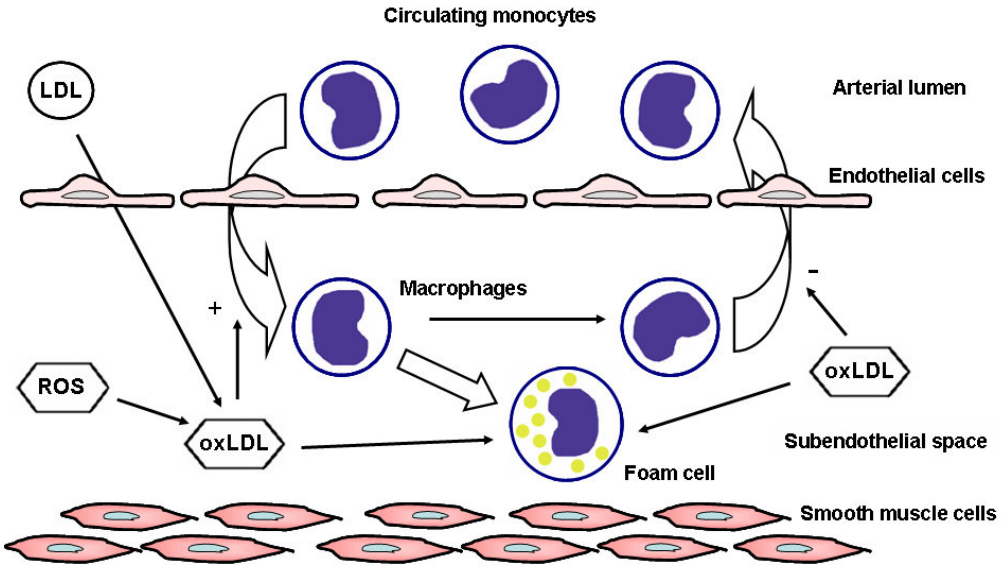

Figure 1.5 Oxidative modification hypothesis of atherogenesis.

Circulating monocytes in the bloodstream are drawn into the subendothelial space and become macrophages. Native LDL is taken up and oxidised by ROS such as O_2^- released from local vascular SMCs, ECs and macrophages. Macrophages take up oxLDL to become foam cells, which are precursors to atherosclerotic development. OxLDL causes endothelial dysfunction and injury which encourages monocyte recruitment and inhibits macrophage regress back into lumen.

transport of lipoproteins into the artery wall to form the fatty streak (102). Oxidative stress, and in particular the activity of the ROS producing enzyme, NADPH oxidase, is an important contributor to coronary plaque instability, which can trigger acute coronary syndromes by leading to plaque rupture and resulting in thrombotic occlusion of the artery (103).

1.3.2 Antioxidants and cardiovascular disease

In response to numerous different ROS found in biological systems, cells have evolved to develop potent defensive antioxidant strategies. Endogenous antioxidant enzymes include SOD, catalase, glutathione peroxidase and glucose-

6-phosphate dehydrogenase. Non-enzymatic antioxidants include glutathione, ascorbate (vitamin C), α-tocopherol (vitamin E) and high-density lipoprotein (HDL).

Given that oxidative stress is a hallmark of many cardiovascular disease processes, much work has been done to determine if oral supplementation of antioxidants can prevent clinical events such as myocardial infarction and cardiovascular death. Some early trials showed promising results. In addition to its antiplatelet and anti-inflammatory actions, Vitamin E reduces the oxidation of LDL and is perhaps the most widely tested antioxidant therapy in clinical studies (104). The Cambridge Heart Antioxidant Study (CHAOS) randomised 2 002 patients with proven CHD to oral vitamin E or placebo and demonstrated a reduction in the primary endpoint of cardiovascular death or non-fatal myocardial infarction (105). However, the encouraging results of CHAOS have not been confirmed in larger randomised studies. In the 9 541 patient Heart Outcomes Prevention Evaluation (HOPE) trial, vitamin E failed to reduce cardiovascular events in patients with high cardiovascular risk (106). Similarly, negative results were reported using Vitamin E in the post-myocardial infarction GISSI-Prevenzione trial (107) and the Heart Protection Study (HPS), which investigated the use of a combination of antioxidant vitamins for primary and secondary prevention (108).

The failure of systemic antioxidants to reduce cardiovascular events in clinical trials has been discussed widely and some authors speculate on an inability to target therapies to population subtypes with enhanced oxidative stress such as

diabetics or end-stage renal disease (109). Others suggest that more potent antioxidants are required to demonstrate clinical benefit (110). Crucially, very few studies have evaluated oxidative status at a cellular level in treated patients, instead opting to supplement antioxidants without confirming antioxidant efficacy. Nevertheless, on the basis of current evidence, oral antioxidant supplementation is not recommended for the prevention of cardiovascular events. The local delivery of antioxidants directly to the vascular wall represents an opportunity to achieve high concentration at the intended site of action with relatively few side-effects, although this technique is currently poorly studied.

1.3.3 Oxidative stress and PCI

Evidence for increased oxidative stress following PCI

Inflation of an angioplasty balloon to high atmospheric pressure to dilate a stenotic coronary artery with deployment of a metal stent causes extensive mechanical trauma to the artery wall, resulting in marked vessel injury. As a result, the artery releases a large amount of ROS.

Ex-vivo rabbit artery rings subjected to balloon injury demonstrated immediate release of ROS as a result of vessel wall NADPH oxidoreductase activity (111). The release was proportional to degree of injury and not prevented by prior removal of the endothelium or antagonism of other ROS generating sources such as XO, NOS or mitochondrial electron transport. Another study demonstrated that *ex-vivo* porcine coronary artery segments continue to release large amounts of O_2^- for at least several days after *in vivo* balloon injury (112). O_2^- production was essentially abolished after pretreatment of the artery ring with the NADPH

oxidase inhibitor, diphenyleneiodonium (DPI), again underlining the predominance of this enzyme in ROS generation after balloon injury.

A method for *in vivo* estimation of oxidative stress as a result of PCI in humans involves simultaneous assay of coronary sinus blood, which provides venous drainage for the heart. Compared to upstream left main coronary artery blood, isoprostanes (end-products of ROS mediated-lipid peroxidation) are increased (113). Coronary angioplasty in humans also causes an acute increase in plasma levels of oxidised lipoproteins (114;115). The acute increase in oxidative stress following PCI may be partly responsible for impaired microcirculatory function after stent deployment. In a study of stable angina patients, the administration of intravenous ascorbic acid was shown to decrease the release of oxidative biomarkers (8-hydroxy-2-deoxyguanosine and 8-iso-prostaglandin F_{2alpha}) and improve angiographic evidence of myocardial perfusion following elective PCI (116), although no differences in clinical outcome were detected.

The influence of oxidative stress on cellular processes after PCI

It is clear that oxidative stress is enhanced after arterial injury and, by influencing multiple signalling pathways of vascular repair, it appears to play an important role in the regulation of NIH (117). Early transient exposure to oxidised glutathione, a recognised consequence of oxidative stress, is capable of amplifying cell proliferation and NIH, which is sustained for weeks after arterial balloon injury and mediated through a redox-active metal-dependent pathway (118). The activation of the transcription factor nuclear factor κB (NF-κB) is enhanced by redox processes after injury (111) and this may have a lasting effect

on the gene programme involved in NIH, including expression of vascular cell adhesion molecule-1 (VCAM-1), MCP-1 and other pro-inflammatory factors (119;120). ROS also serve as second messengers to activate many intracellular proteins and enzymes, including the epidermal growth factor receptor (EGFR), c-Src, p38 mitogen-activated protein kinase (MAPK), Ras and Akt/PKB, which suggests a significant role in the regulation of vascular SMC growth and migration, modification of extracellular matrix and modulation of EC function (121). The response of vascular SMCs to PDGF, which includes tyrosine phosphorylation, MAPK stimulation, DNA synthesis and chemotaxis, is at least partly dependent on elevated intracellular levels of the ROS, H_2O_2 (122). The prominent influence of oxidative stress on NIH after vascular injury is matched by a major involvement in the regulation of endothelial recovery. OxLDL formed by the oxidative modification of native LDL in the bloodstream is widely present in coronary artery lesions. Atherosclerotic plaques enriched with high levels of oxLDL are more likely to become unstable (123), and plasma oxLDL is acutely elevated after PCI (115). oxLDL is toxic to EPCs (124), which are likely to suffer from impaired antioxidant defences in the presence of atherosclerosis (125). oxLDL is also a potent inhibitor of EC migration and this occurs via an O_2^- dependent mechanism (126). As a result, oxLDL may influence healing of a stent deeply embedded in coronary lesions so that the rate of endothelialization is suboptimal, even after BMS. The inhibitory effect of oxLDL on EC migration in culture is blocked by the antioxidant vitamin E (127). Uptake of the lipophilic antioxidants probucol and vitamin E, both stimulate EC proliferation in culture (128) and are protective against the harmful effects of other ROS (129). NO released by the endothelium encourages migration and growth of ECs (130), and

endothelial NOS (eNOS) expression is increased in regenerating endothelium following injury (131). However, in situations where there is major oxidative stress, such as PCI, there may be widespread scavenging of NO by released ROS, such as the reaction of NO with O_2^- to form $ONOO^-$, one of the most potent endocellular oxidants (132). The protective effect of NO on endothelial regeneration may be lost and, instead, $ONOO^-$ produced, which is harmful to ECs (133). These findings support a role for antioxidant therapy delivered to the site of arterial injury to defend the regenerating endothelium against the deleterious effects of oxLDL, O_2^-, $ONOO^-$ and other toxic ROS, whilst allowing NO to establish a protective effect on the endothelial healing process.

Inhibition of thrombus formation and promotion of endothelial regeneration after PCI is highly preferable to reduce the risk of stent thrombosis. The underlying pathophysiology of arterial thrombosis is intrinsically linked to oxidative stress (134). Primarily, ROS inactivate NO, which has important antiplatelet actions (32). Vitamin E supplementation in healthy individuals has been shown to inhibit platelet aggregation (135). It has also been shown to decrease oxidative stress, increase platelet NO release, delay intra-arterial thrombus formation (136) and improve fibrinolysis via a reduction in plasminogen activator inhibitor activity (137). The antioxidant, N-acetyl-cysteine has been shown to reduce the procoagulant response following aortic balloon injury by decreasing bound thrombin activity and platelet adhesion (138). Other antioxidant compounds have been shown to inhibit platelet aggregation. A series of caffeic acid anilides exhibited potent anti-oxidative activities and inhibited arachidonic acid-induced platelet aggregation (139). Pomegranate juice, a natural source of antioxidants,

inhibited human platelet aggregation in response to collagen (140). Antioxidant flavonoids from black tea were found to decrease platelet aggregation in healthy human subjects (141). DPI, an inhibitor of NADPH-oxidase, also inhibits platelet aggregation (142).

Despite the relative wealth of *in vitro* and animal data implying the importance of oxidative stress and the ability of some antioxidants to influence critical molecular and cellular vascular pathophysiological processes, less impressive results have been witnessed in human studies with no consistent evidence that these compounds have the capacity to influence hard clinical outcomes such as myocardial infarction or cardiovascular death.

1.3.4 Antioxidants and PCI

Much of the experimental data supporting the role of antioxidants to inhibit NIH and improve endothelial repair has been reinforced by several *in vivo* studies suggesting that strategies to decrease oxidative stress can reduce restenosis and encourage re-endothelialization after PCI (Table 1.1). As the major source of ROS after balloon injury is via NADPH oxidase, inhibitors of this enzyme have been tested and found to reduce NIH in animal models (143;144). Delivery of extracellular SOD gene therapy to the artery wall after balloon denudation of the rabbit aorta reduced NIH and improved endothelial recovery (145). Another study in atherosclerotic rabbit iliac arteries showed that gene transfer of SOD and catalase dramatically reduced ROS release from the artery wall after balloon angioplasty (146). This was accompanied by less neointimal inflammation and

Table 1.1 Main antioxidant studies for restenosis and re-endothelialisation.

Study (ref)	Anti-oxidant	Species and dose	Main findings
Jacobson *et al.* (143)	NOX inhibitor	Rat carotid balloon injury IP minipump	Reduced NIH ($p<0.05$)
Dourron *et al.* (144)	NOX inhibitor	Rat carotid balloon injury Gene transfer	Reduced NIH ($p<0.05$)
Laukkanen *et al.* (145)	SOD	Rabbit aortic balloon injury Gene transfer	Reduced NIH, improved re-endothelialisation (both $p<0.001$)
Durand *et al.* (146)	SOD/CAT	Rabbit iliac balloon injury Gene transfer	Reduced restenosis, improved re-endothelialisation (both $p<0.05$)
Schneider *et al.* (147)	Probucol	Pig coronary balloon injury Oral 2 g/day	Reduced NIH ($p=0.007$)
Miyauchi *et al.* (148)	Probucol	Rabbit carotid balloon injury Oral 1.3 g/day	Reduced NIH ($p<0.05$)
Lau *et al.* (149)	Probucol	Rabbit aortic balloon injury Oral 0.75% wt/wt	Reduced NIH, improved re-endothelialisation (both $p<0.05$)
Tanous *et al.* (150)	Probucol	Rabbit aortic stent Oral 1% wt/wt	Reduced NIH and restenosis (both $p<0.05$), improved re-endothelialisation ($p=0.008$)
Tardif *et al.* (151)	Probucol	Human PTCA Oral 1 g/day	Reduced restenosis (improved remodelling) ($p=0.003$) and repeat PTCA ($p=0.009$)
Yokoi *et al.* (152)	Probucol	Human PTCA Oral 1 g/day	Reduced restenosis ($p<0.01$)
Sekiya *et al.* (153)	Probucol	Human coronary stent Oral 500 mg/day	Reduced restenosis rate (17% vs 32%, p=NS), with cilostazol ($p<0.05$)
Kim *et al.* (154)	Probucol	Human coronary stent Oral 500 mg/day	Reduced restenosis rate (21% vs 24%, p=NS)
Wakeyama *et al.* (155)	Probucol	Human coronary stent Oral 500 mg/day	Reduced NIH (<0.001) and reduced restenosis ($p=0.1$)
Tardif *et al.* (156)	Probucol	Human coronary stent Oral 1 g/day	Increased follow up LA ($p<0.05$) due to greater acute gain
Nunes *et al.* (157)	Probucol	Human coronary stent Oral 1 g/day	No effect on NIH or restenosis
Kim *et al.* (158)	Probucol	Pig coronary DES Stent delivery	No effect on NIH or restenosis
Tardif *et al.* (156)	AGI-1067 (succinobucol)	Human coronary stent Oral 280 mg/day	Increased follow up LA ($p<0.05$) due to greater acute gain
Kim *et al.* (158)	Carvedilol	Pig coronary DES Stent delivery	Reduced NIH ($p=0.004$) and reduced restenosis ($p=0.002$)

NOX, NADPH oxidase; IP, intraperitoneal; CAT, catalase; PTCA, percutaneous transluminal coronary angioplasty; LA, lumen area.

reduced restenosis, with improved re-endothelialization and endothelial function. However, there is no data yet to suggest that incorporation of gene transfer to reduce restenosis in humans is a viable option and therefore these studies have poor applicability. Most techniques to deliver gene-based therapy during angioplasty rely on infusion of active treatments directly into the artery wall and therefore have significant practical limitations, compared with DES. Despite some evidence that NO encourages EC growth *in vitro*, L-arginine (a NO precursor) had no effect on re-endothelialization in denuded rabbit iliac arteries, although it effectively inhibited neointimal growth (159).

Early trials of probucol administration in animal models of balloon angioplasty demonstrated a reduction in NIH and restenosis (147;148). Probucol restricts the severity of NIH by inhibiting vascular SMC proliferation via enhanced G1/S-phase growth arrest and improving endothelial healing (149). A major mechanism by which probucol mediates these beneficial effects is believed to be via up-regulation of heme oxygenase-1, which induces SMC apoptosis (by producing heme breakdown products carbon monoxide, biliverdin and bilirubin) and promotes EC function (160). In keeping with these findings, supplementation of probucol inhibited NIH and improved re-endothelialisation in a rabbit model of aortic balloon injury (149). Owing to the positive animal studies, probucol has now been extensively tested in clinical trials, perhaps more than any other antioxidant compound. The Multivitamins and Probucol (MVP) trial and Probucol Angioplasty Restenosis trial (PART) both reported a clear reduction in restenosis after coronary angioplasty (151;152). However, a subsequent IVUS substudy of the MVP trial showed that the beneficial effect of probucol was

related to improved post-angioplasty remodelling, rather than suppression of NIH (161).

Investigation in the stent era has continued with a variety of clinical trials examining the effects of oral probucol on ISR. Oral probucol approximately halved ISR rates in one clinical trial, although the effect was only significant when the drug was combined with the phosphodiesterase inhibitor, cilostazol (153). A trend to inhibit ISR was reported in another clinical study (162); however, the trial design was criticised due to inadequate preloading of oral probucol, which accumulates slowly in tissues and is usually commenced four weeks before the procedure. A clinical trial designed to determine the effect of candesartan and probucol on ISR found no benefits from angiotensin receptor blockade; however, using probucol, there were significant reductions in angiographic late loss and NIH by IVUS analysis (155).

The large Canadian Antioxidant Restenosis Trial (CART-1) was a multi-centre, double-blind, placebo-controlled randomised trial in 305 patients comparing oral administration of probucol, succinobucol (AGI-1067) or placebo after PCI (156), involving stents in 85% of patients. Succinobucol is a novel probucol derivative with superior pharmacokinetic, antioxidant and anti-inflammatory properties compared with probucol (163-165). It has been shown to prevent atherosclerosis in animal models (164;166) and is discussed in more detail in Chapter 5. Two weeks prior to PCI, patients were assigned to one of the 5 treatment groups (probucol 500 mg twice daily, succinobucol 70 mg, 140 mg, 280 mg once daily or placebo) and treated for 2 weeks before and 4 weeks after PCI. Angiographic

follow-up with baseline and 6-month IVUS examinations was performed to evaluate the efficacy of each group. Positive results were reported, with greater stent luminal area present in the highest dose of succinobucol and probucol groups, compared with placebo after 6 months. There was a significant dose-response relationship for succinobucol, suggesting a true biological effect. Although CART-1 is often cited to provide evidence that succinobucol and probucol are both capable of reducing ISR, no statistically significant difference was demonstrated for this end-point unless compliant patients were analysed separately. Furthermore, detailed IVUS analysis showed clearly that supplementation of neither oral succinobucol nor probucol caused inhibition of neointimal growth and the advantages in final luminal area were due to greater acute luminal gain at the time of the index PCI. The underlying mechanism for this effect was not clear but speculatively may have been related to improved endothelial function of the diseased segment or favourable alterations in plaque composition due to antioxidant pre-treatment, allowing greater luminal gain at the time of balloon dilation and stent deployment. CART-2 was a subsequent IVUS study originally designed to investigate the effect of oral succinobucol 280 mg once daily on ISR following PCI, however due to considerable difficulties with the technical quality of serial IVUS examinations, the trial was altered to examine the effect of succinobucol on coronary plaques in adjacent segments. Although atherosclerosis regression was evident in the succinobucol group, this effect was not significantly different from placebo (167). Taking the data from the CART-1 and CART-2 clinical studies and that of the ISAR-TEST clinical research program into consideration, succinobucol is unlikely to be an efficacious oral antirestenotic agent in clinical practice but may provide

advantageous if successfully incorporated in a fully biocompatible DES or potentially as a dual coating with a recognised inhibitor of restenosis (such as rapamycin) to improve its performance, similar to the probucol/rapamycin dual DES, and this strategy appears to merit further exploration.

In more recent clinical study, oral probucol failed to reduce ISR following PCI, as determined by IVUS (157). This result should be viewed with some caution because confounding factors known to increase the risk of ISR were present in the probucol group, such as the treatment of significantly smaller vessels and a higher incidence of diabetes and acute lesions. A rabbit study which looked specifically at the effects of probucol on healing after stent injury reported a considerable improvement in stent endothelialization, accompanied by a reduction in NIH and less evidence of stent thrombosis (150). In this trial, oral probucol therapy also reduced leucocyte accumulation around the stent struts, suggesting its anti-inflammatory effects may exert a significant role in suppression of NIH, at least in animals.

These studies testing the ability of antioxidants to reduce restenosis after balloon angioplasty or stenting have generally relied on oral administration. Studies which consistently demonstrated the most impressive reductions in ISR were performed in animals, where oral regimens are possible at a dose not possible in clinical practice. The application of stent-based delivery of antioxidants may provide an ideal opportunity to achieve optimal local tissue concentration, whilst minimizing toxic systemic side-effects. Such work has been performed with carvedilol, a β-blocker with potent antioxidant activity. It has been shown that

implantation of a carvedilol-coated stent in a porcine model was capable of inhibiting ISR and providing complete re-endothelialization (158). The same study failed to demonstrate a beneficial effect from a probucol-coated stent, although there were concerns about adequate drug delivery in this study.

Given the broadly favourable preclinical data for high-dose oral antioxidant supplementation after coronary angioplasty and the modest effect of oral antioxidants evident in humans, the challenge of future trials is likely to focus on the incorporation of novel potent antioxidants into contemporary DES technology, which will provide targeted delivery and increased local tissue concentration without the potential drawbacks of systemic toxicity and administration, mirroring the successful development of other potent antiproliferative agents delivered via DES.

1.4 Preclinical models of restenosis

Several animal models have played a pivotal role in preclinical studies to examine the biological events resulting from PCI and to test the efficacy and safety of stents prior to investigation in human studies.

1.4.1 Murine models

Murine models of arterial injury and restenosis are favoured by cost-effectiveness and ease of handling and housing, as well as providing a relatively accessible way to examine the influence of a wide variety of molecular biomarkers. The rat carotid model for restenosis was developed following

extensive use as a model for human atherosclerosis. Whilst these models have provided extensive data on the effects of endothelial denudation on SMC proliferation and inflammatory responses (168), the cumulative shortcomings of the murine models are significant. The effects of arterial injury cause a modest neointimal response in rodents and critically, results in these models have failed to consistently replicate the effect of test compounds in human studies, most notably the effect of angiotensin-converting enzyme inhibitors (169).

1.4.2 Hypercholesterolaemic rabbit iliac model

The rabbit iliac model has been used extensively to study atherosclerosis and restenosis. Rabbits are readily susceptible to dietary increases in cholesterol which leads to rapid atherosclerotic development, which is similar but not identical to that found in humans. Important differences exist in the histological composition and behaviour of atheromatous lesions in the rabbit model compared with humans, leading to limitations in the translation of data into clinical studies. An important criticism of this model for assessment of restenosis therapies is that the abundant foam cells and extracellular matrix generated by the hypercholesterolaemic state are not heavily involved in human neointimal formation. Nevertheless, despite these caveats, the rabbit iliac stenting model remains a relatively cost-effective way to assess the efficacy of antiproliferative drugs and has provided valuable data on the relative safety and toxicity of DES in the past decade (85;170).

1.4.3 Porcine coronary artery model

Early pig models were able to demonstrate that the acute trauma related to coronary angioplasty was responsible for severe injury and provocation of the restenotic cascade in humans (171;172). The response of healthy porcine coronary arteries to overstretch injury closely replicates that found in human restenosis. Stent injury in the porcine coronary artery leads to thick neointimal development within 28 days, identical to human restenotic tissue. Coronary artery anatomy is similar to humans and therefore allows the use of human catheterisation equipment to accurately reflect clinical practice. The flow dynamics of the porcine coronary vasculature closely parallels that of humans, enabling a more reliable estimate of drug pharmacokinetics to take place. The disadvantages of the pig model are higher costs compared to smaller animals with related housing practicalities and expenses. Other considerations in the pig are potential differences in the inflammatory reactions to stent injury, which may display excessive granulomatous or eosinophilic infiltrates (173). Crucially, the effects of DES in the porcine model have been most predictive of their effects in humans, providing the strongest rationale for testing novel devices in this species (174). The pig coronary artery model has therefore become the standard model for the preclinical evaluation of DES (175).

1.5 Hypothesis and aims

The current literature indicates that oxidative stress may play a substantial role in modifying platelet and cellular responses following PCI. The events leading to ISR and stent healing are complicated and not completely understood, but

oxidative stress appears be involved in a number of important aspects. This thesis will investigate the effect of oxidative stress on several key processes closely involved in the response to arterial injury and the ability of antioxidants to favourably modify vascular healing. Specific approaches to decrease the impact of ROS will be tested, although it is appreciated that focussing on one particular antioxidant strategy may be insufficient to totally eliminate the effects of oxidative stress during vascular repair, as other oxidative pathways may remain active. As this may limit efficacy after PCI, complementary antiproliferative strategies in addition to a purely antioxidant approach may be considered.

The first part of the thesis will focus on *in vitro* aspects occurring immediately after stent deployment. The effect of abundant ROS released after PCI may directly promote blood aggregation which could contribute to a higher risk of early stent thrombosis, the period at greatest risk because the stent has not yet endothelialised. Enhancement of platelet aggregation in human whole blood by locally released oxidants may increase the risk of myocardial infarction, carrying a substantial risk of death. This thesis will therefore examine the direct effect of ROS on blood aggregation and also whether ROS influence the antithrombotic properties of the endothelium, which is responsible for rapidly recovering the stent surface and providing protection against stent thrombosis. The direct effect of ROS on the migration of healthy ECs will also be investigated, as this may indicate that locally increased oxidative stress has the capacity to impair endothelialisation of stents, thereby exacerbating the risk of stent thrombosis and also promoting the rapid growth of neointimal tissue, which is known to be inhibited by regenerated endothelium.

In vivo testing will provide the basis for the second part of this thesis. I will evaluate the ability of a novel potent antioxidant loaded on a DES to offer improved healing and reduced neointimal thickening in a preclinical model of restenosis, using the pig coronary artery overstretch injury method. This is likely to provide valuable information on the potential for antioxidant DES to outperform other novel DES in clinical studies. The highly biocompatible polymer-free microporous Yukon® stent platform will be used to avoid the negative impact of persistent polymers on arterial healing and minimise the likelihood of late "catch-up" restenosis or aggravated inflammation. This stent platform will also enable "in-house" individualised DES coating and is already supported by large-scale clinical research studies (ISAR program, Munich Heart Centre). Scarce data exists reporting the effectiveness of an antioxidant-eluting stent approach in a preclinical or clinical research setting. I hypothesise that delivery of a suitable, potent antioxidant from a polymer-free DES may be able to achieve local suppression of oxidative stress, thus inhibiting platelet aggregation and neointimal growth, and accelerating re-endothelialisation, with minimal systemic toxicity (figure 1.6). These attributes may reduce the risk of ISR and stent thrombosis.

The final part of the thesis will investigate more broadly the influence of circulating ROS on another important aspect of stent healing and the risk of thrombosis. As described already, it is now recognised that regrowth of functional endothelium is supported by the mobilisation and homing of EPCs to sites of endothelial damage. This systemic mechanism of vascular restoration

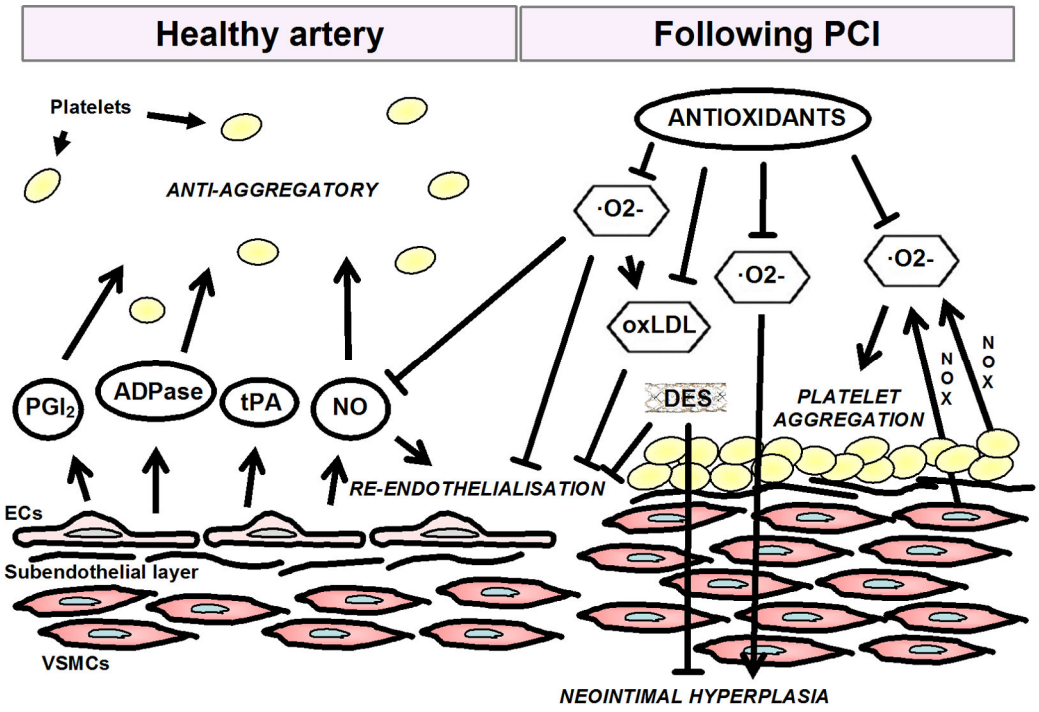

Figure 1.6 The potential role of antioxidants after PCI. The endothelium prevents thrombosis via the actions of prostacyclin (PGI_2), ADPase, tPA and NO, prevents contact between blood and the subendothelial layer (rich in procoagulant materials), and inhibits NIH. First-generation DES inhibit NIH, but impair re-endothelialisation. PCI injury causes major O_2^- release (primarily via NOX), which impairs re-endothelialisation and promotes NIH. Antioxidants may limit NIH and reduce thrombosis by accelerating re-endothelialisation and scavenging O_2^-.

may constitute an important component of re-endothelialisation after arterial injury secondary to PCI (176). The relationship between systemic levels of oxidative stress in the bloodstream, which is elevated due to PCI, on this endothelial repair system is not well known. The potential for circulating EPCs to maintain coronary endothelial function in patients with CHD is also poorly recognised. This thesis will therefore investigate the correlation between plasma levels of oxLDL (a major component of oxidative stress in humans), the number of circulating EPCs and endothelial function in patients with CHD. Patients with

stable angina already scheduled for PCI will be recruited to facilitate invasive testing of their coronary endothelial function at the time of their planned coronary intervention and also to provide access to patient blood from a population with significant CHD undergoing PCI.

To summarise, the overall aims of this thesis are:

1 Using human whole blood and cultured porcine ECs, to investigate the influence of oxidative stress and antioxidants on WBA, the EC-platelet interaction and EC migration.

2 Using an *in vivo* model of porcine coronary stent implantation, to determine whether a novel antioxidant-eluting stent leads to reduced NIH and improved healing.

3 In patients with angina, to determine whether plasma levels of oxLDL are associated with impaired endothelial repair (reduced circulating EPCs), impaired coronary endothelial function or increased whole blood aggregation.

Chapter 2

The effect of oxidative stress on whole blood aggregation and the endothelial cell-platelet interaction

2.1 Introduction

Oxidative stress is recognised as an important mediator of atherothrombotic events in cardiovascular disease (134). The classic paradigm is the formation of platelet-rich thrombus overlying a region of injured or dysfunctional endothelium. In addition to many other factors, oxidative stress participates in the regulation of platelet activation and thrombus formation. The effects of ROS on thrombus formation are of direct clinical relevance to PCI, which is known to stimulate the release of ROS from the surrounding vascular wall. The direct effects of ROS on platelet function are reportedly varied. Previous platelet assays in healthy individuals have reported both pro- and anti-aggregatory effects when platelets are exposed to exogenous ROS (177). Platelets themselves also generate ROS and this appears to be of significant relevance during recruitment, adhesion and aggregation (178).

Although there is good evidence that oxidative stress damages the endothelium *in vivo*, which predisposes individuals to thrombosis, the close interactions between ROS and ECs are not completely understood. Healthy ECs inhibit thrombosis (179), but the direct actions of ROS on ECs may influence their antithrombotic properties, which may elevate the risk of stent thrombosis.

WBA offers the most physiological setting in which to examine platelet-mediated thrombus formation. Furthermore, it closely replicates the milieu in which clinical thrombosis occurs, where other blood constituents including erythrocytes (180), leucocytes (181) and plasma-derived substances (182;183)

are involved. ECs are thought to inhibit platelet aggregation by release of NO (32), prostacyclin (33), and the activity of endothelial ectonucleotidases (184), although in the presence of heightened oxidative stress, some of these effects may be deficient. My aims were to quantify the effects of ROS on WBA and to assess the influence of ROS on the EC-platelet interaction in patients with CHD.

2.2 Methods

2.2.1 Chemiluminescence

Lucigenin chemiluminescence (CL) was used in non-biological samples to confirm generation of ROS (specifically superoxide anion, O_2^-) as a consequence of the X/XO reaction (185). 900 µl phosphate buffered saline (PBS) was added to a clear plastic sample cuvette. This was prewarmed at 37 ºC for 5 minutes in an incubation chamber. 100 µl lucigenin solution (Sigma-Aldrich, Dorset, UK) was prepared in distilled water and added to the cuvette with the test substance(s). As lugenin may itself interfere with O_2^- generation (186), a range of lucigenin concentrations were tested and the concentration producing the least additional chemiluminescence signal was used for subsequent experiments. The cuvette was transferred immediately to a chemiluminometer (Berthold, Germany). The delay was set to 10 seconds and the chemiluminescence signal was recorded after 120 seconds in relative light units per seconde (RLU/s). Xanthine (Sigma-Aldrich, Dorset, UK) was dissolved in 10 mM NaOH and XO (Sigma-Aldrich, Dorset, UK) was dissolved in PBS.

2.2.2 Study population for WBA

This study was approved by the West Glasgow and University of Strathclyde ethics committees. All participants were provided with a Participant Information Sheet (PIS) and gave their informed written consent. Preliminary WBA experiments were performed on venous blood donated by healthy human volunteers, recruited from the University of Strathclyde cardiovascular laboratory. In order to examine the effect of oxidative stress in a population closely matched to those undergoing PCI, adult patients with symptomatic stable CHD who were referred to the Western Infirmary Glasgow for out-patient cardiology appointments were recruited. All patients were over age 18 and receiving chronic oral aspirin therapy. Patients were excluded if there was a history of myocardial infarction within 3 months, if they were unable to give informed consent or if they were taking any other antithrombotic therapy.

2.2.3 Whole blood aggregometry

After recruitment, venous blood was withdrawn into vacuum filled plastic 3.5 ml Vacuette® tubes (Greiner Bio-One, Austria) containing 3.2% sodium citrate (0.109 mol/L). Four Vacuette® tubes (approximately 14 ml) of whole blood were collected from each donor or patient. Unless used to assess WBA over time, blood was tested within 3.5 hours of venepuncture. WBA was measured using an impedance aggregometer (Chrono-log, Model 590) and impedance was recorded using a dual channel chart recorder (Kipp & Zonen, BD 41). 500 µl citrated whole blood was diluted 1:1 with 500 µl normal saline to a total volume of 1 ml in a plastic sample cuvette. A stir bar was added to the cuvette, placed in the device reaction well and prewarmed for 5 minutes at 37 ºC, with a stirring speed

of 900 rpm. An electrode containing two fine palladium wires was inserted. The platelets in the whole blood adhered to the wires, forming a uniform platelet monolayer. A small voltage difference was applied across the wires, and the electrical impedance was measured. In the absence of aggregating agonist, the impedance between the two wires became constant after 2 minutes, producing a stable baseline which constituted zero. The aggregometer was then calibrated to 20 ohms (Ω) equivalent to 40 mm deflection using the chart recorder controls. When an agonist was added to the cuvette, platelets in the blood were activated and started to aggregate, coating the palladium wires on the electrode and causing a corresponding increase in electrical impedance. This change in impedance was directly proportional to the extent of aggregation and was measured in Ω on the chart recorder set at 2 cm/min and followed for 5 minutes after addition of agonist. Collagen and adenosine diphosphate (ADP) (Labmedics Limited, Manchester, UK) were used as platelet agonists in all experiments. For X/XO experiments, individual aggregation values were calculated as a percentage of aggregation compared to control conditions, for both agonists and blood was incubated with the test substance(s) for 1 minute prior to addition of each agonist.

2.2.4 EC culture

Freshly removed pig hearts were obtained from a local abattoir within 2 hours of slaughter. The pulmonary artery was removed and pinned to a Sylgard coated block. Using a sterile scalpel blade, porcine pulmonary artery ECs were scraped gently from the luminal surface of the main pulmonary artery, under a sterile hood. ECs were transferred to a 15 ml tube of large vessel EC growth medium

package (basal medium, growth supplement and antibiotic [TCS Cellworks, Buckinghamshire, UK]). The cell suspension was centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed and the ECs were resuspended in 1 ml fresh growth medium. This was added to T-25 cell culture flasks (Nunc, Kamstrupvej, Denmark) with additional growth medium and cultured in an incubator at 37 ºC in a humidified atmosphere of 5% CO_2. Growth medium was replaced every two days and ECs were transferred to T-75 flasks when 90% confluence was reached. Fresh growth medium was prepared once weekly. All ECs were used from passages 2 to 4.

2.2.5 EC-platelet interaction

ECs were collected from the flasks by removal of growth medium and addition of 2 ml TrypLE Express (Invitrogen Corporation, Paisley, UK) to detach the cells. Following 15 minutes, the cell suspension was transferred to centrifuge flasks and spun in a centrifuge (Mistral 1000, MSE centrifuge) at 10,000 rpm for 5 minutes. The supernatant was removed and the pellet of cells resuspended in 1 ml blank EC growth medium. The number of ECs in 1 ml suspension was counted using a haemocytometer. 1 x 10^5 ECs were added to the sample cuvette using an appropriate volume of cell suspension and made up to 500 µl with normal saline, to which 500 µl whole blood was added. In control experiments, an equivalent volume of blank growth medium was added to the cuvette in place of cell suspension. WBA was tested as previously described and the effect of ECs was determined in the absence and presence of X/XO and following 5 minutes pre-treatment of ECs with 100 µM N_ω-nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich, Dorset, UK), an inhibitor of NOS.

2.2.6 Statistical analysis

The SD of pilot WBA data under control conditions was found to be 25%. A sample size of 14 patients was calculated to provide 80% power to detect a treatment difference at a two-sided 0.05 significance level, if the true difference between groups was 40%. All data are expressed as mean ± SEM unless otherwise stated. Groups were compared by independent samples t test or repeated measures analysis of variance and post-hoc Dunnett's test. Statistical analysis was performed using the SPSS statistical software package 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

2.3 Results

2.3.1 Lucigenin concentration-response

The effect of 5 µM, 50 µM and 250 µM lucigenin on chemiluminescence was examined. This represents a range of concentrations which have the potential influence baseline ROS production and redox-recycling, however minimal effects are previously reported for 5 µM (185). 5 µM lucigenin in the absence of X/XO caused very low chemiluminescence (62 ± 2 RLU/s). In the presence of 100 µM X and 10 mU/ml XO, both higher doses of lucigenin (50 µM and 250 µM) caused significantly increased chemiluminescence, compared to 5 µM lucigenin ($p < 0.001$) (Figure 2.1). 5 µM lucigenin was therefore used for all subsequent chemiluminescence experiments, as this gave the least assay interference, as previously confirmed (185).

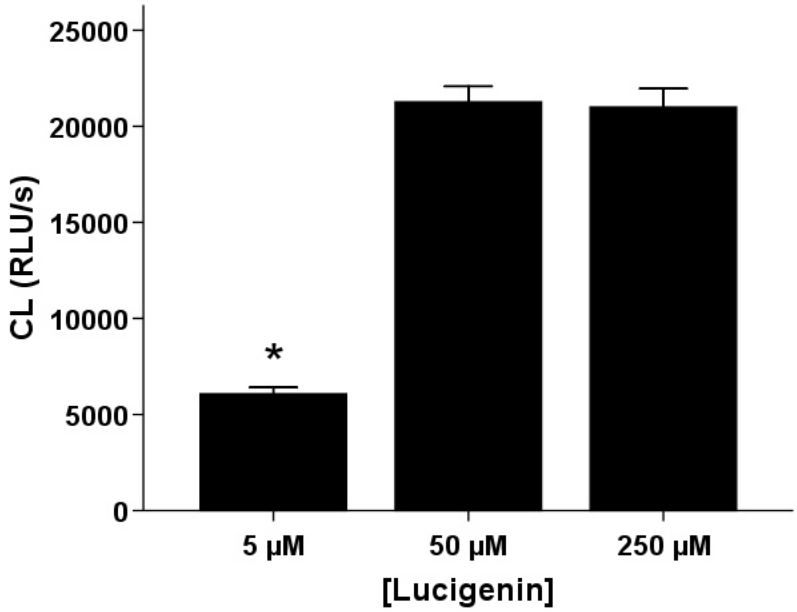

Figure 2.1 The effect of lucigenin on chemiluminescence. Chemiluminescence (CL) using different concentrations of lucigenin, in the presence of a constant concentration of 100 µM X and 10 mU/ml XO (n = 4), * $p < 0.001$ vs. other groups.

2.3.2 Confirmation of O_2^- production by X/XO

There was a highly significant positive correlation (r = 0.94, p = 0.006) between chemiluminescence and XO concentration in the presence of a constant concentration of 100 μM X (Figure 2.2). 50 U/ml SOD completely abolished chemiluminescence in the presence of 100 μM X and 10 mU/ml XO ($p < 0.001$), indicating that O_2^- was the principal active molecule generated under the conditions of these experiments (Figure 2.3).

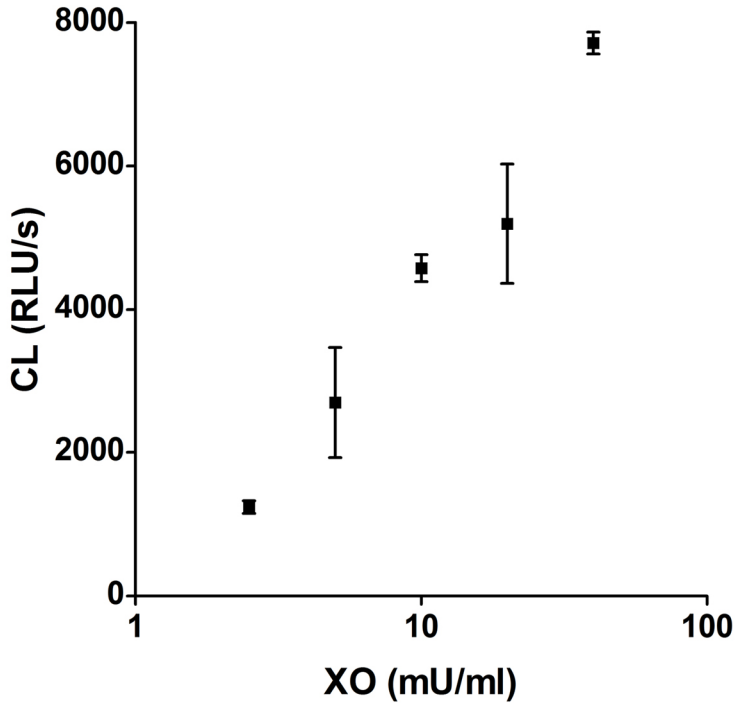

Figure 2.2 The effect of X/XO on chemiluminescence. CL due to increasing XO concentration (log 10 scale) in the presence of a constant concentration of 100 μM X (n = 2), r = 0.94, p = 0.006.

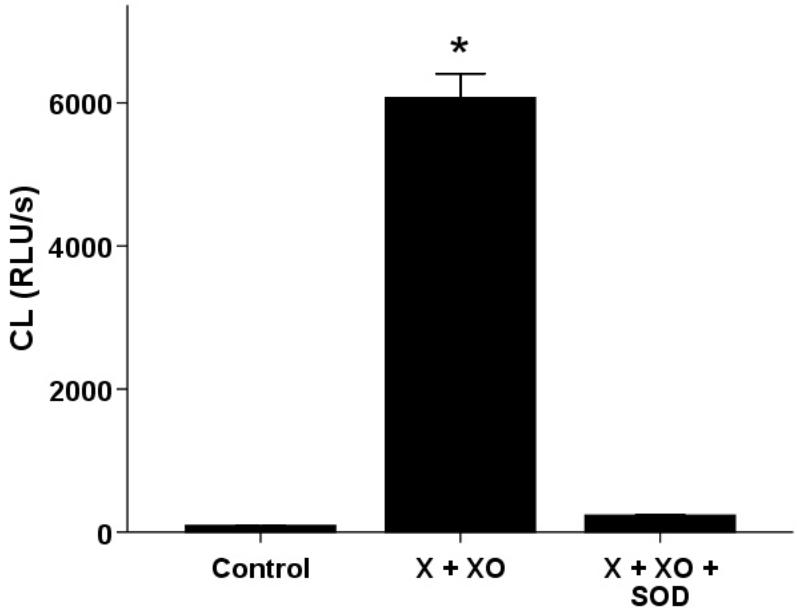

Figure 2.3 The effect of SOD on X/XO-induced chemiluminescence. CL due to 100 μM X and 10 mU/ml XO with the effect of 50 U/ml SOD (n = 4-8), * $p < 0.001$ vs. control.

2.3.3 Baseline characteristics of WBA participants

Venous blood was withdrawn from 12 healthy donors and used for preliminary experiments. 8 (67%) donors were male and the mean age was 30.5 years (SD 9.2). No donor had a significant past medical history or was taking regular medication. The baseline characteristics of 23 recruited patients are shown in Table 2.1. There was a high prevalence of additional cardiovascular risk factors and regular medications prescribed. As patients were tested on the day of recruitment, all patients were taking regular aspirin. No patient was taking clopidogrel or any other antithrombotic medication.

Table 2.1 Baseline characteristics of WBA patients

	Prevalence, (n = 23)
Patients' characteristics	
Age, mean (SD), y	62.7 (8.9)
Male, n (%)	17 (73.9)
Current smoker, n (%)	6 (26.1)
Hypertension, n (%)	11 (47.8)
Hypercholesterolaemia, n (%)	15 (65.2)
Diabetes mellitus, n (%)	10 (43.4)
Family history of premature CHD, n (%)	8 (34.8)
Previous myocardial infarction, n (%)	9 (39.1)
Previous stroke, n (%)	1 (4.3)
Previous PCI or CABG, n (%)	7 (30.4)
Multivessel disease, n (%)	13 (56.5)
Preserved LV function, n (%)	18 (78.3)
Heart failure, n (%)	3 (13.0)
Drug treatment	
Aspirin, n (%)	23 (100.0)
Clopidogrel, n (%)	0 (0.0)
Statin, n (%)	22 (95.7)
ACE inhibitor or ARB, n (%)	17 (73.9)
Beta-blocker, n (%)	17 (73.9)
Calcium channel blocker, n (%)	9 (39.1)
Diuretic, n (%)	5 (21.7)
Nitrate, n (%)	17 (73.9)
Nicorandil, n (%)	11 (47.8)

2.3.4 WBA intra-individual variability

Preliminary evaluation was performed on a single donor blood sample to assess the intra-individual variability of WBA. 6 experiments were performed within one hour of venepuncture using 3 μg/ml collagen as agonist. Agreement between replicates was excellent, as shown in Figure 2.4 (mean 13.67 Ω, SD 0.5164).

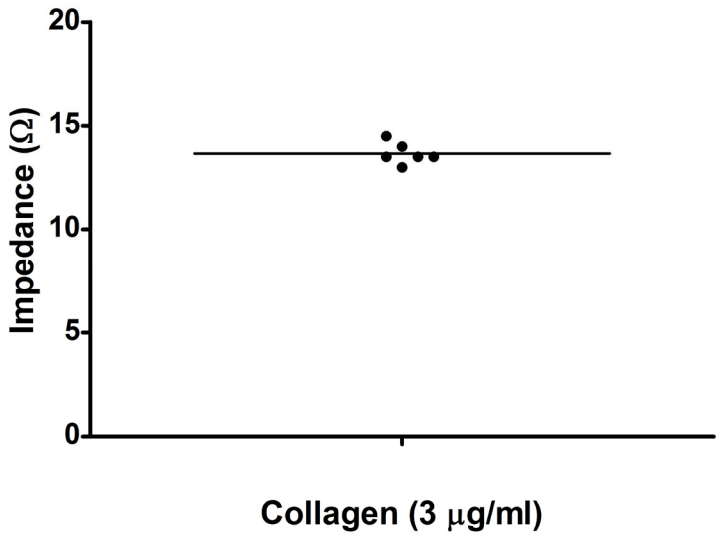

Figure 2.4 Intra-individual variability of WBA (n = 6).

2.3.5 WBA agonist concentration-response

A range of collagen and ADP concentrations were tested to assess the concentration-response for each agonist, using donor blood. The EC_{50} for collagen was found to be 3 μg/ml (Figure 2.5) and for ADP was 5 μM (Figure 2.6). The EC_{50} agonist concentrations were used for all subsequent experiments to study the potential of ROS to modify aggregation in either direction without the limitation of testing near minimal or maximal conditions. Therefore, although maximal impedance values for each agonist were different due to their different

activation mechanisms, agonist concentrations were matched for the level of activation of their respective receptors.

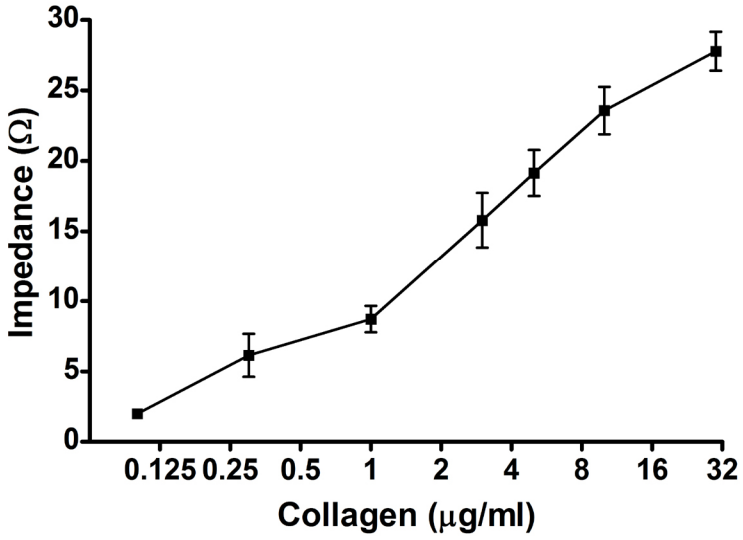

Figure 2.5 WBA concentration-response curve for collagen (n = 7).

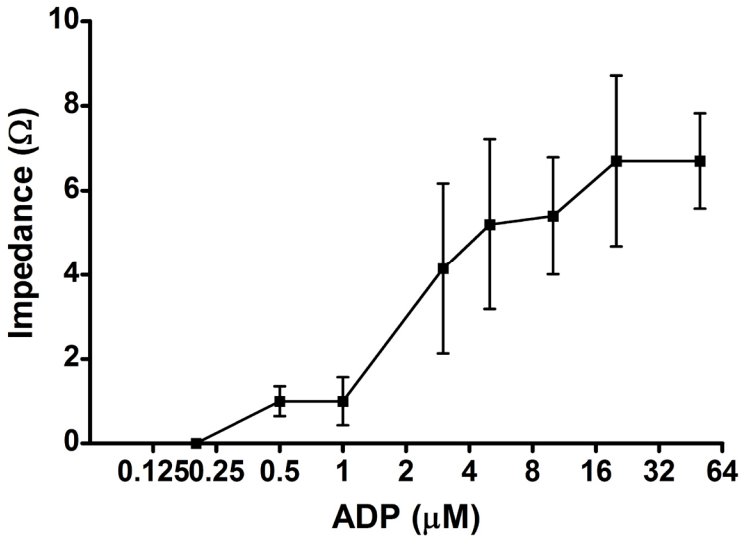

Figure 2.6 WBA concentration-response curve for ADP (n = 5).

2.3.6 WBA time-response

Due to the serial nature of experiments performed on each blood sample, I assessed the variation in WBA over time, for each agonist. WBA was tested under identical conditions using donor blood at intervals of up to 6 hours following venepuncture. There was a slight trend for collagen-induced WBA to decrease after 6 hours although this was not statistically significant (Figure 2.7). ADP-induced WBA showed minor variation over time, although there was no significant change in aggregation between 1 and 4 hours (Figure 2.8).

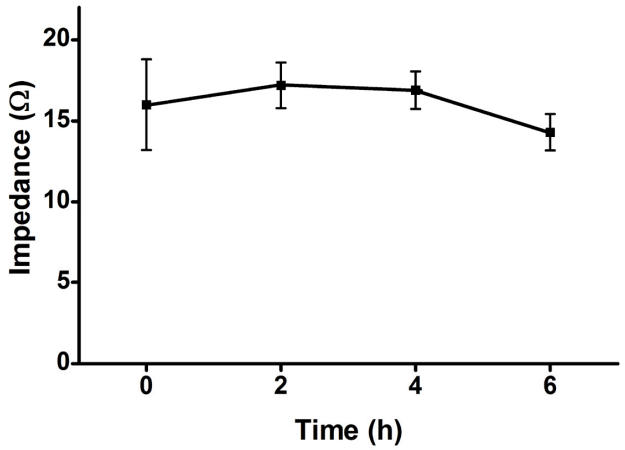

Figure 2.7 WBA time-response curve for collagen (3 µg/ml, n = 5), p = NS.

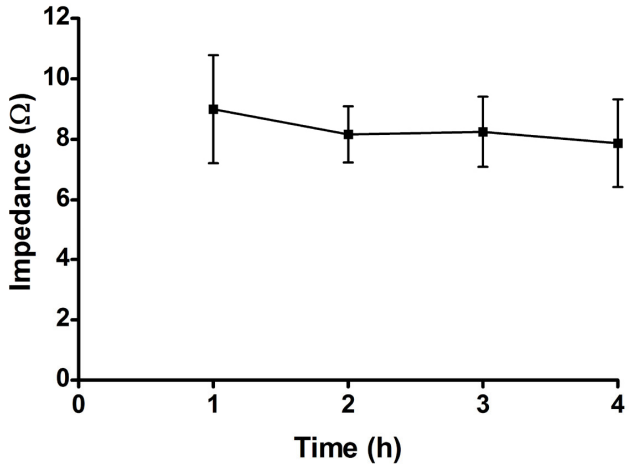

Figure 2.8 WBA time-response curve for ADP (5 µM, n = 4), p = NS.

2.3.7 Comparison of WBA between donors and patients

There was no significant difference in WBA between donor blood and patient blood, for each agonist. Under identical control conditions, WBA was 15.9 ± 1.5 Ω for donor blood (n = 12) compared with 15.4 ± 0.8 Ω for patient blood (n = 23) in response to 3 µg/ml collagen (p = NS). WBA was 7.8 ± 1.5 Ω for donor blood (n = 10) compared with 9.3 ± 0.9 Ω for patient blood (n = 23) in response to 5 µM ADP (p = NS).

2.3.8 Effect of X/XO on WBA

The effect of oxidative stress on WBA in 14 CHD patients was determined using the X/XO reaction. The concentration of X was kept constant at 100 µM, whereas the concentration of XO was tested at 10, 50 and 100 mU/ml to engender incremental levels of oxidative stress, previously confirmed using chemiluminescence. ROS produced by X/XO caused a concentration-dependent inhibition of WBA in CHD patients compared to control, for both collagen (Figure 2.9) and ADP (Figure 2.10). 100 µM X and 100 mU/ml XO inhibited WBA in response to collagen by 28.9% (95% CI 15.9% - 41.8%, $p < 0.001$) and in response to ADP by 36.0% (95% CI 9.6% - 62.4%, $p = 0.005$). The effect in patients with diabetes mellitus did not differ significantly from the effect in patients without diabetes mellitus (n = 7, data not shown). A smaller number of identical experiments were performed using healthy donor blood, which also demonstrated concentration-dependent inhibition of WBA, which was significant at the highest dose of XO (n = 4, data not shown).

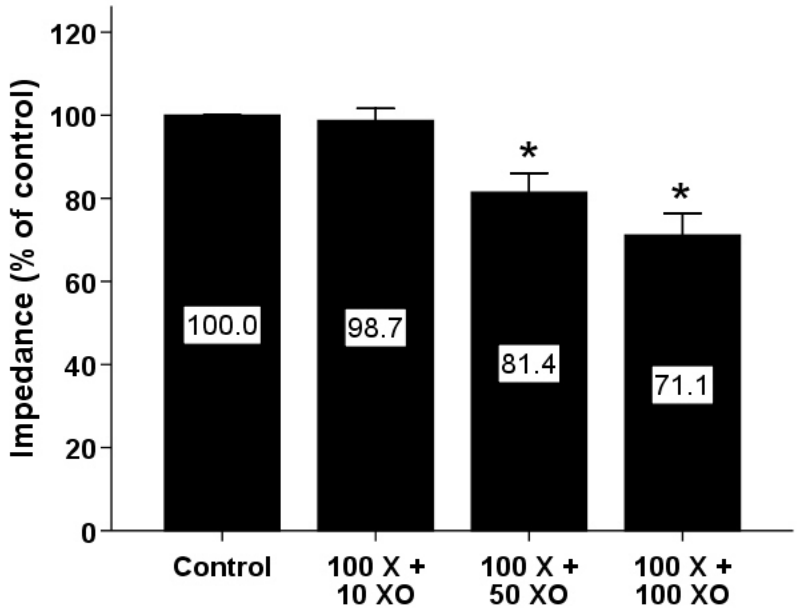

Figure 2.9 The effect of X/XO on WBA in response to collagen. X (µM) + XO (mU/ml) caused a concentration-dependent inhibition of WBA in response to 3 µg/ml collagen in CHD patients (n = 14), * $p < 0.05$ vs. control.

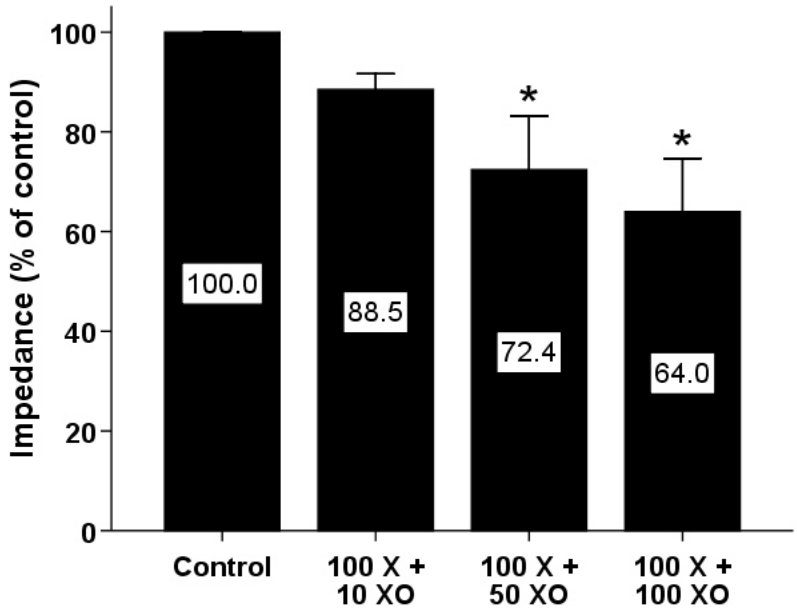

Figure 2.10 The effect of X/XO on WBA in response to ADP. X (µM) + XO (mU/ml) caused a concentration-dependent inhibition of WBA in response to 5 µM ADP in CHD patients (n = 14), * $p < 0.05$ vs. control.

2.3.9 EC-platelet interaction

The effects of ECs on WBA in 9 CHD patients are shown in Figures 2.11 and 2.12. The addition of 1 x 10^5 cultured ECs caused a significant decrease in WBA in response to collagen by 31.2% (95% CI 12.2% - 50.2%, $p < 0.01$) and ADP by 31.6% (95% CI 2.5 - 60.7%, $p < 0.05$). The inhibitory effect of ECs on WBA remained significant after pre-treatment of ECs with the combination of 100 µM X and 10 mU/ml XO, in response to collagen (29.1% [95% CI 10.1% - 48.1%, $p < 0.01$]) and ADP (32.6% [95% CI 3.4 - 61.7%, $p<0.05$]). However, pre-treatment with 100 µM L-NAME attenuated the inhibitory effect of ECs, which was no longer statistically significant in response to either collagen (21.6% [95% CI -7.2% - 50.5%, p = NS]) or ADP (9.2% [95% CI -44.7% - 63.1%, p = NS]).

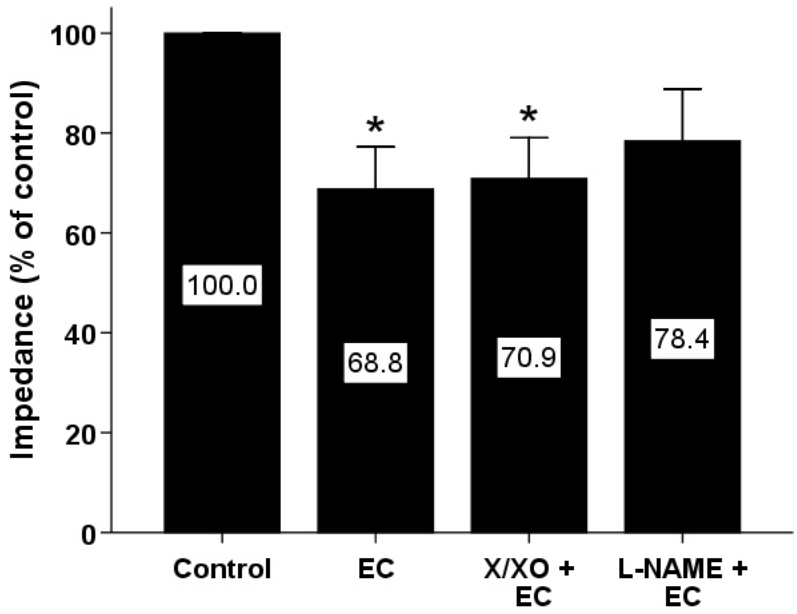

Figure 2.11 The effect of ECs alone and after pretreatment with X/XO or L-NAME on WBA in response to collagen. Cultured ECs inhibited WBA in response to 3 µg/ml collagen. This effect was not affected by 100 µM X and 10 mU/ml XO but was no longer significant after pre-treatment of ECs with 100 µM L-NAME (n = 9), * $p < 0.05$ vs. control.

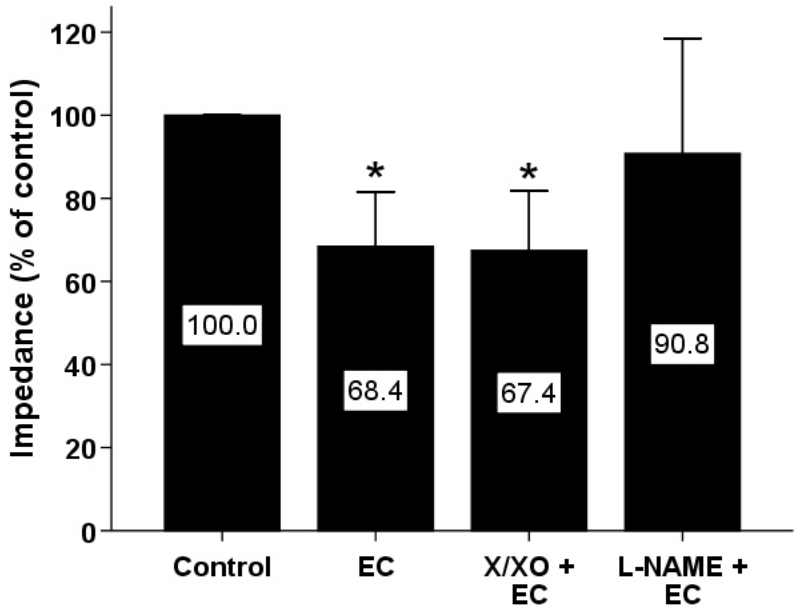

Figure 2.12 The effect of ECs alone and after pretreatment with X/XO or L-NAME on WBA in response to ADP. Cultured ECs inhibited WBA in response to 5 μM ADP. This effect was not affected by 100 μM X and 10 mU/ml XO but was no longer significant after pre-treatment of ECs with 100 μM L-NAME (n = 9), * $p < 0.05$ vs. control.

2.4 Discussion

After arterial injury or atherosclerotic plaque rupture, circulating platelets adhere to the damaged vessel wall and become activated by agonists in the extracellular matrix such as collagen, von Willebrand factor and fibrinogen. Other agonists such as ADP, thrombin, adrenaline and platelet activating factor potentiate the platelet response. Once activated, platelets release granules containing cytokines and growth factors which lead to a cascade of events resulting in platelet aggregation, formation of a platelet plug and in some cases vessel occlusion, causing a myocardial infarction or stroke. Recently, evidence has accumulated

that ROS are heavily involved in the regulation of platelet function, although their precise role is complex (177).

Clarifying the actions of ROS on WBA in patients with symptomatic CHD extends our understanding of the pathophysiology of acute thrombosis, which in patients who have undergone PCI is associated with a very high mortality (38). This chapter investigated the direct effects of oxidative stress on human whole blood, which has direct relevance to PCI as large amounts of ROS are released from the artery wall during this period and may be implicated in stent thrombosis. The greatest influence of ROS on the stent-vessel wall interaction is likely to be derived from locally generated ROS as a result of arterial injury (principally via NOX) but also from elevated systemic levels of circulating ROS, which are increased in patients with CHD.

2.4.1 Preliminary results

My initial experiments confirmed that the X/XO reaction was generating ROS within the time frame of WBA. The potential interference of lucigenin used in these experiments was minimal, due to the very low concentration used, which has been shown to exert only minor effects on O_2^- production (185). 5 µM lucigenin was associated with very low chemiluminescence in my experiments. I demonstrated that the WBA assay was reliable and consistent, with a low intra-individual variability. All samples were tested within a time frame that I have shown to have no impact on WBA. Notably, WBA was not significantly different between healthy donors and patients. I propose that the administration of chronic aspirin therapy negated the increased platelet aggregation likely to be present in

CHD patients compared with healthy individuals on no antithrombotic therapy. The lack of difference supported the translation of preliminary methodological data to the patient population.

2.4.2 Effect of ROS on platelet aggregation

One of the primary purposes of the current study was to examine, for the first time, the effects of exogenous ROS on WBA in patients with clinically proven CHD. My results have shown that ROS, generated by the X/XO reaction, cause a concentration-dependent inhibition of WBA in these patients. This reduction in aggregation is demonstrated in response to the biologically relevant agonists, collagen and ADP. Whilst I have confirmed using chemiluminescence that O_2^- is generated by X/XO, this reaction also leads to the downstream production of the highly reactive molecule, H_2O_2 (187), which plays a significant role in the modulation of platelet function. Additionally, although resting platelets do not produce NO, intracellular NO is formed following stimulation with platelet agonists (188), and this can react with O_2^- to produce $ONOO^-$ (132), a potent oxidant and additional contributor to platelet aggregation (189). I propose that these secondary molecules may have played a major role in the inhibition of WBA in these experiments.

The reported effects of ROS on platelet function are variable and depend on a variety of factors, especially the type and concentration of ROS and the milieu in which it acts. Using washed platelets in buffer, exposure to ROS generated by X/XO led to irreversible aggregation to subthreshold levels of ADP, although there was no effect on collagen-induced platelet aggregation (190). In another

study, collagen (but not ADP or thrombin) induced O_2^- release from platelets, which stimulated ADP-dependent platelet recruitment to preformed thrombus, but did not in itself cause platelet aggregation (190). Others have shown that addition of X/XO to washed platelets led to release of the surrogate marker serotonin, thought to occur as a result of platelet aggregation, whereas exposure of platelets to X/XO also lowered the threshold for thrombin-induced aggregation (191). This effect was blocked by SOD suggesting that O_2^- was the relevant molecule involved. The O_2^- generator pyrogallol increased thrombin-induced platelet aggregation, an effect which was also blocked by SOD (192), and increased arachidonic acid-induced platelet aggregation, an effect which was inhibited by dipyridamole (193). Put together, these data suggest the more likely effect of O_2^- on isolated platelets is pro-aggregatory, albeit mild in some cases.

Importantly, the addition of X/XO to platelets in platelet rich plasma (PRP) has led to different results compared to those found in isolated platelets. In healthy volunteers, X/XO led to potent inhibition of platelet aggregation in response to ADP, collagen and U-46619 (a thromboxane mimetic) (194). These findings were attributed to the abolition of the second wave release reaction with an overproduction of H_2O_2, stimulation of guanylate cyclase and an increase in cGMP. This inhibitory effect on platelet aggregation was blocked by catalase, but not by SOD, which suggested that H_2O_2 was the crucial inhibitory ROS. The inhibitory effect of H_2O_2 on ADP-induced aggregation has been confirmed in other studies (195;196). There have been reports that lower concentrations of H_2O_2 have the capacity to enhance subthreshold collagen and arachidonic acid-induced aggregation, but not ADP-induced aggregation (197;198). The influence

of $ONOO^-$ on isolated platelets has been varied, causing either pro-aggregatory (199) or anti-aggregatory effects (200;201), but studies in PRP show that $ONOO^-$ consistently inhibits collagen, ADP, and thrombin-induced aggregation (199;202). Subsequent reports suggested that the NO-dependent inhibitory effects of $ONOO^-$ occurred at lower concentrations than the pro-aggregatory effects (203).

Taking all of these data into consideration, the actions of ROS on platelet function are diverse, depending on the molecule involved, and concentration-dependent. Furthermore, assessment of platelet function must take into account the environment in which aggregation is stimulated. Although washed platelets suspended in buffer can provide information on the specific effects of substances on isolated platelets, a more physiological environment can be simulated in plasma or whole blood, which allow other blood constituents and endogenous antioxidants to be represented which may antagonise the stimulation of platelets induced by ROS (204).

The key finding from my results is that oxidative stress (generated by X/XO) caused a concentration-dependant inhibition of platelet aggregation in whole blood derived from patients with CHD. I propose that this was caused by the generation of secondary inhibitory ROS such as H_2O_2 and $ONOO^-$, possibly related to endogenous substances present in whole blood. The main goal of future studies would be to confirm the precise species involved, the mechanism and the importance of this effect with reference to the levels of oxidative stress present in clinical situations such as PCI. A closer estimate of the potential

mechanism of this effect could be assessed by studying the effects of SOD given prior to X/XO, thereby removing the effects of O_2^- and causing generation of H_2O_2. Catalase could be used to specifically assess the loss of H_2O_2 from the system. In addition, other specific scavengers of secondary ROS including $ONOO^-$ could be used to clarify the influence of these molecules.

2.4.3 Effect of ROS on the EC-platelet interaction

I demonstrated that healthy ECs maintain the ability to inhibit WBA derived from patients with CHD. ECs are thought to inhibit thrombosis predominantly through the actions of NO, prostacyclin (205) and the activity of an endothelial ecto-ADPase present on the surface of ECs, identified as CD39 (206;207). It was reported that depolarization of ECs led to O_2^- production, which inactivated the ecto-ADPase, thereby decreasing the inhibitory properties of ECs on ADP-induced aggregation, (208). Therefore it was expected that ROS would at least partly neutralise the inhibitory effect of healthy ECs on WBA by opposing the effects of NO and/or endothelial ecto-ADPase. However, I have demonstrated that the addition of exogenous ROS using X/XO failed to influence the inhibitory properties of healthy ECs. This may have resulted from an overall equilibrium of negative and positive effects on blood aggregation. The X/XO reaction may have produced ROS such as O_2^-, which inactivated NO and endothelial ectonucleotidases (actions that would promote aggregation), but also produced H_2O_2 and $ONOO^-$, leading to inhibitory effects. To explore this further, I have shown that the inhibitory effect of healthy ECs is decreased by the pretreatment of ECs with L-NAME. This supports the notion that isolated removal of EC-derived NO from the blood decreases its anti-aggregatory capacity, which

implies an increased risk of thrombosis where endothelial dysfunction and reduced NO bioavailability occurs.

2.4.4 Limitations

Well recognised limitations exist concerning the translation of this work to the clinical field. It is unclear how the addition of exogenously derived ROS relates to the extent and nature of ROS release following PCI. The main source of ROS after PCI is generation via NOX, the output of which may differ significantly from the X/XO reaction. The effects of ROS on blood aggregation have been investigated in isolation and subsequently in the presence of healthy ECs, rather than in the presence of damaged endothelium and the presence of a foreign body, as is present after coronary stenting. We must accept that the applicability of these *in vitro* studies to the clinical scenario of early stent thrombosis may be limited. Porcine rather than human ECs were used to replicate the effect of human endothelium due to cost constraints, but it seems unlikely that the effect would have been significantly different if human ECs had been used.

2.4.5 Conclusions

ROS generated by X/XO produce a concentration-dependent inhibition of WBA in patients with CHD. This concurs with previous studies in healthy individuals using X/XO as a source of ROS and is likely to be a result of highly reactive molecules such as H_2O_2 and $ONOO^-$, which share inhibitory properties that appear to predominate over O_2^-, which tends to promote aggregation. Healthy ECs maintain their physiological role to inhibit platelet-derived thrombus

formation in blood derived from atherosclerotic human populations, even after short term exposure to ROS. This suggests that strategies to preserve endothelial function and improve re-endothelialisation are likely to be beneficial after PCI. However, persistent endothelial dysfunction or patchy endothelial regrowth due to persistent drug or polymer effects after PCI is likely to diminish the capacity of the endothelium to generate NO, which could put patients at greater risk of stent thrombosis.

Chapter 3

The effect of oxidative stress on endothelial cell migration

3.1 Introduction

After PCI, the migration of ECs to reconstitute the denuded endothelium is a crucial process that occurs early after vessel trauma. In the rat carotid balloon injury model, the endothelium regenerates at approximately 2-3 mm per week (209), depending on the degree of injury, with more severe cases taking longer. Deeper medial injury encourages more SMCs to migrate to the neointima, which inhibit the migration and proliferation of ECs in that area (210). Endothelial regrowth is specific to the species involved. For instance, stent healing in pigs is slower than in rats but still occurs around six times faster than in humans (174). Identifying the factors controlling the movement of these vital cells is paramount to understanding the mechanisms that contribute to ISR. Besides a central role in modulating vascular tone and haemostasis, regeneration of confluent endothelium provides an impermeable barrier between medial SMCs and circulating growth factors. ECs also produce a number of growth-promoting and growth-inhibitory factors which regulate SMC proliferation. Healthy ECs maintain the quiescence of SMCs by releasing NO (211). Hence, regeneration of the endothelium significantly influences the extent of neointimal formation.

ECs themselves are stimulated to a lesser degree, or inhibited, by many substances that stimulate SMC migration, such as angiotensin-II (212), serotonin, noradrenaline and IL-1 (213). Factors that promote EC migration include VEGF (214), platelet-derived EC growth factor (PD-ECGF) (215), oestradiol (216), oxytoxin (217), ACE inhibitors (212), heparins (218), endothelins (219), thrombin (220), tPA (221), high density lipoprotein (222) and shear stress, which

increases EC migration onto metallic stent surfaces (223). NO appears to exert a biphasic effect on EC migration. Acute release of NO, generated by eNOS, reportedly promoted EC migration towards angiogenic factors, such as VEGF (224), endothelin (130) and substance P (225). NO appeared to exert its stimulatory effect on EC migration through activation of soluble guanylyl cyclase mechanisms (226). On the other hand, others have observed that chronic basal endogenous NO release inhibited EC migration (227), an effect which was matched by other NO donors (228). Since the recognition that endothelial recovery may reduce ISR and lower the risk of stent thrombosis, more attention has been re-focussed on revascularisation devices which might accelerate EC regrowth (229;230). The effect of ROS on EC migration is not well defined and, given that oxidative stress is a major consequence of PCI, requires further evaluation. Therefore, the aim of this study was to investigate the effect of ROS generated by X/XO on EC migration, using a scratch wound assay. The effect of the antioxidant, probucol, on EC migration would also be evaluated.

3.2 Methods

3.2.1 EC culture

The method for EC cell culture is described in detail in section 2.2.4. Briefly, porcine pulmonary artery ECs were removed from fresh pig hearts and cultured in large vessel EC growth medium. ECs were maintained at 37°C in a humidified atmosphere of 5% CO_2, and used from passages 2 to 4. After obtaining sufficient quantities, the ECs were collected from T-75 flasks using TrypLE Express and seeded in 6 well plates.

3.2.2 EC migration assay

EC migration was measured by the scratch wound method as described by Bürk (231). Once grown to confluence in 6 well plates, ECs were made quiescent by exchanging the serum-containing growth medium with serum-free medium, followed by the addition of 1 mg/ml gelatin. The ECs remained in serum-free medium for 24 hours. Following this, a wound was made in the EC monolayer by pressing a rigid razor blade down on the well. The blade was gently drawn across the base of the well, scraping the ECs to one side (Figure 3.1). Care was taken to avoid reusing a damaged blade which had developed gaps in the cutting edge, otherwise lines of ECs would remain attached to the well, perpendicular to the wound. After a successful razor scrape, the detached cell debris and medium were aspirated and disposed of. Serum-free medium with 1 mg/ml gelatin was replaced and the test substance(s) was added using a pipette. The ECs were incubated for a further 24 hours. For each well, 2 adjacent regions consisting of 1,300 µm lengths of razor wound in the centre of the microscopic field were examined and the number of cell nuclei that had crossed the starting line produced by the razor scrape was counted. Well plates were fixed and stained using modified Wright-Giemsa stain. Images of migrating ECs were captured using an inverted microscope (Nikon) and attached digital camera (CoolSNAP™-Pro, Media Cybernetics). Representative photomicrographs have been enhanced digitally for graphical purposes.

3.2.3 Cell treatments

EC migration was tested under control conditions, after exposure to oxidative stress using different X and XO concentrations with and without the antioxidant,

probucol, and with probucol alone. Control cells were always treated with the relevant vehicles alone. X and XO were dissolved in 10 mM NaOH and PBS, respectively and probucol was dissolved in PBS.

3.2.4 Statistical analysis

All data are expressed as the mean number ± SEM of ECs which had migrated across the 1.3 mm starting line after 24 hours. All experiments were performed in duplicate, on at least 2 different cell isolates. Groups were compared using a t-test or repeated measures analysis of variance with post-hoc Dunnett's test. Statistical significance was confirmed at $p < 0.05$. Statistical analysis was performed using the SPSS statistical software package 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

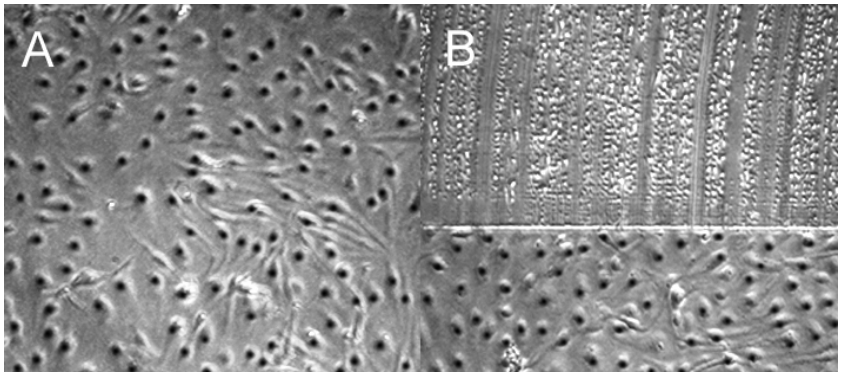

Figure 3.1 Razor scrape EC migration assay. Confluent cultured ECs before (A) and after the razor scrape wound at 0 hours (B).

3.3 Results

3.3.1 General toxicity of X/XO to ECs

Severe generalised toxicity was encountered when ECs were exposed to conditions of oxidative stress, using X/XO. Multiple populations of cultured ECs were exposed to X/XO for 24 hours and, even at relatively low concentrations, ECs detached from the base of the well, rendering the migration assay impossible. ECs remained adherent to the base in the presence of X/XO vehicles. Extensive concentration ranging experiments were performed and eventually it was found that the highest concentration of X/XO which allowed ECs to remain consistently attached to the base of the well was 1 µM X + 0.1 mU/ml XO (Table 3.1). The tolerability of serum-fed ECs to X/XO was considerably better than that of quiescent ECs.

Table 3.1 Toxicity of X/XO to cultured ECs after 24 hours.

Xanthine (μM)	Xanthine oxidase (mU/ml)	Serum medium	Quiescent medium
100	10	×	×
100	2.5	✓	×
100	1	ND	×
10	10	×	×
10	2.5	✓	×
10	1	✓	×
5	2.5	ND	×
2.5	2.5	✓	×
1	10	ND	×
1	2.5	ND	×
1	1	✓	✓/×
1	0.1	✓	✓

Key: ✓ = ECs remained attached to plate

× = ECs detached from plate

✓/× = Variable detachment of ECs

ND = Not done

3.3.2 Effect of low concentration X/XO on EC migration

1 μM X and 0.1 mU/ml XO inhibited EC migration significantly compared with vehicle solutions (11.5 ± 0.5 vs. 27.5 ± 1.0 migrating cells/wound, respectively [$p < 0.01$]), as shown in Figure 3.2. The addition of 20 μM probucol did not influence the effect of X/XO significantly (13.4 ± 0.4 migrating cells/wound, p = NS, probucol vs. X/XO).

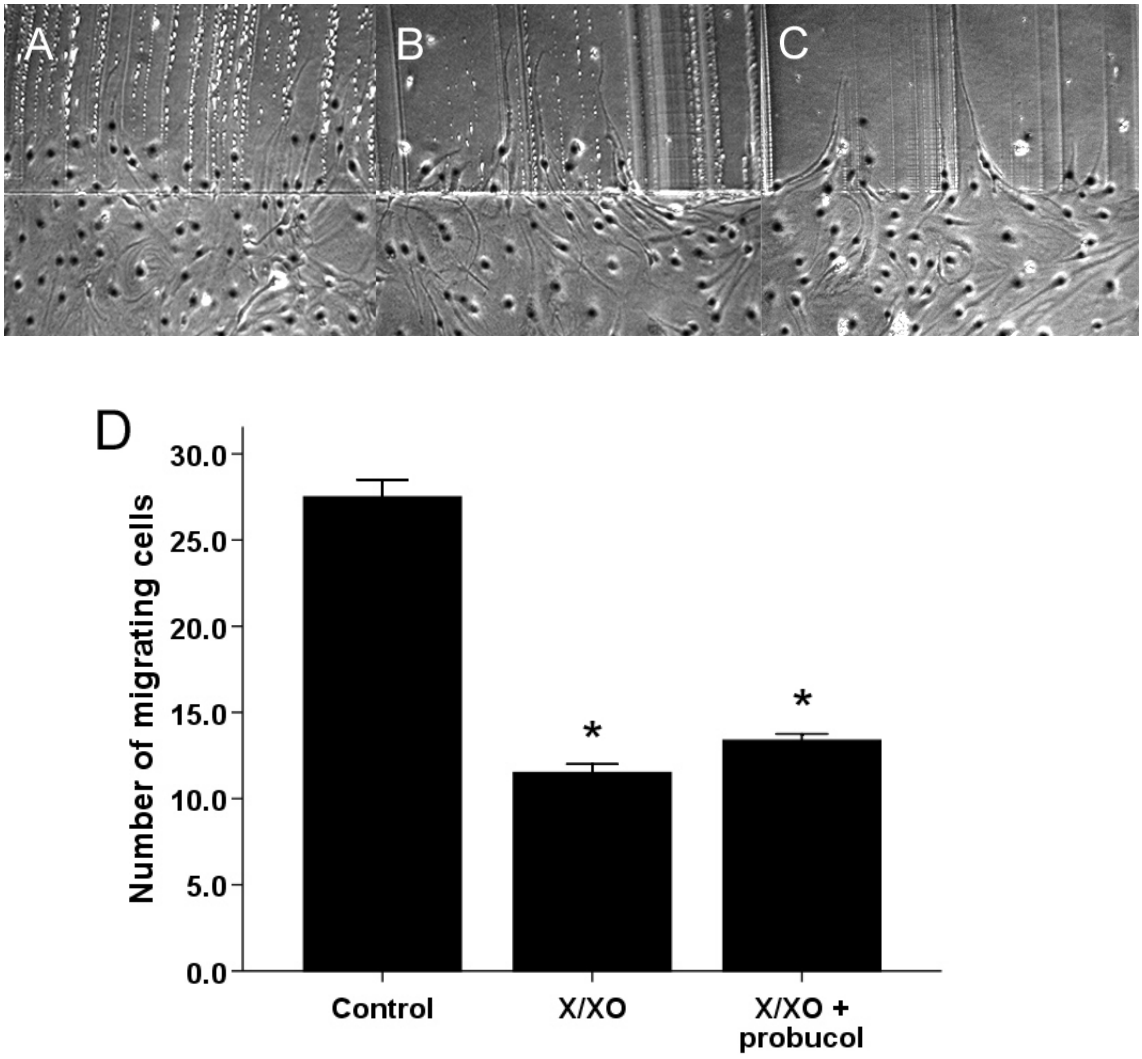

Figure 3.2 The effect of X/XO with and without probucol on EC migration. Representative photomicrographs of control (A), 1 µM X and 0.1 mU/ml XO (B) and 1 µM X + 0.1 mU/ml XO with 20 µM probucol (C) in the scratch wound assay. D, X/XO inhibited EC migration compared with vehicle, an effect which was not altered significantly by 20 µM probucol (n = 2), * $p < 0.01$ vs. control.

3.3.3 Effect of probucol on EC migration

Given that probucol did not appear to protect ECs against X/XO, 20 µM probucol alone was tested to determine whether it would have an effect on EC migration in isolation. There was no significant difference between probucol vehicle and 20 µM probucol (13.4 ± 3.7 vs. 10.4 ± 2.2 migrating cells/wound, respectively, [p = NS]).

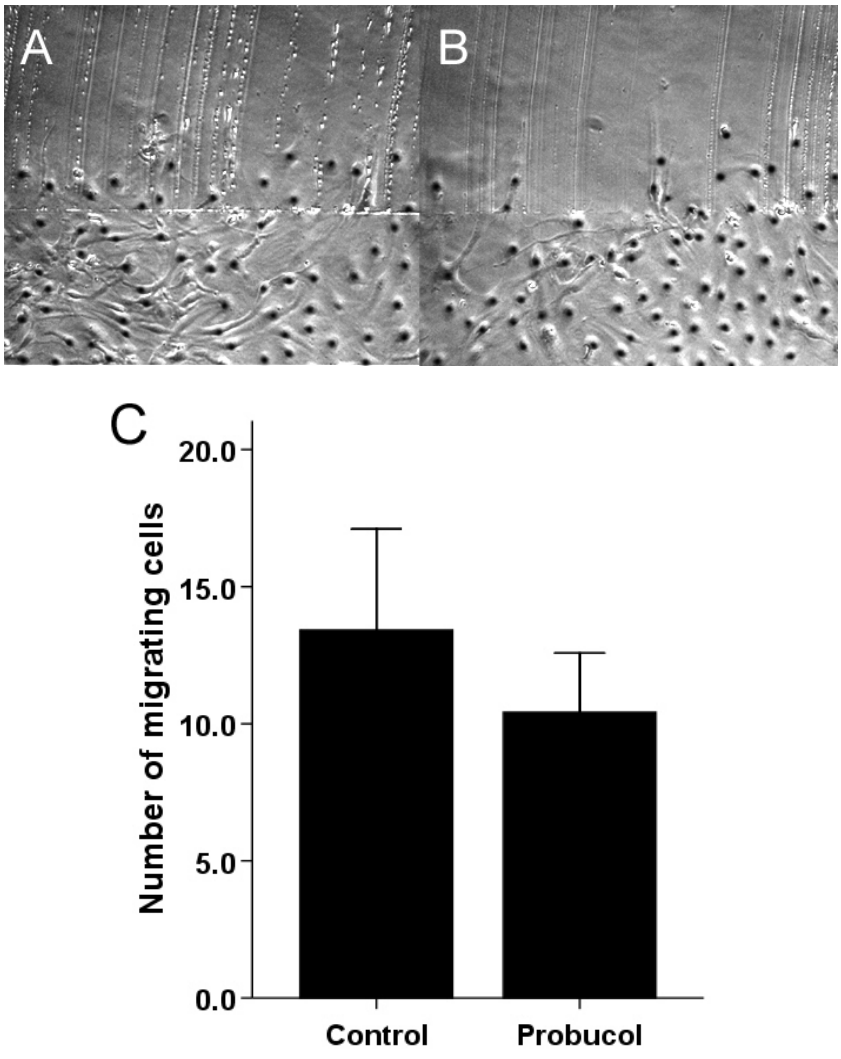

Figure 3.3 The effects of probucol alone on EC migration. Representative photomicrographs of vehicle (A) and 20 µM probucol (B) in the scratch wound assay. C, There was no significant difference between groups (n = 3), p = NS.

3.4 Discussion

3.4.1 General cytotoxicity of oxidative stress to cultured ECs

I found that oxidative stress, applied exogenously using the X/XO reaction, caused severe toxicity to cultured ECs, leading to widespread cell detachment within 24 hours. Direct observation of well plates containing X/XO revealed that detachment started within an hour of exposure to X/XO and was virtually complete within 8 hours. Although I did not directly prove that cell detachment was a consequence of cell death, this was highly probable because floating cells displayed severe morphological changes and general cellular debris was evident on the surface of the culture medium. The cytotoxicity of X/XO was very severe in quiescent ECs, whereas serum-fed ECs had greater tolerance. Toxicity seemed more dependent on XO concentration than xanthine concentration, because serum-fed ECs retained adherence by reducing the XO concentration from 10 mU/ml to 2.5 mU/ml, whereas they showed persistent detachment when xanthine concentration was lowered from 100 µM to 10 µM. I have already demonstrated in Chapter 2 that 100 µM X + 10 mU/ml XO produces significant quantities of ROS. Thus, I propose that a high concentration of ROS in the cell culture medium, released by the X/XO reaction, was responsible for EC death.

3.4.2 Effect of oxidative stress on EC migration

Unfortunately, the migration experiments were severely hampered by the somewhat unexpected severe cytotoxicity of X/XO. Nevertheless, migration of ECs appeared to be significantly inhibited in the presence of exogenous oxidative stress generated by X/XO, within 24 hours. Investigating the underlying

mechanism for this effect fell outside the time frame for this work, however, future experiments could investigate whether ROS generated by X/XO deactivate molecular signalling pathways involved in normal migratory activity. The effects of SOD and inhibitors of NOX may help clarify the specific role of O_2^- in EC migration. Given that EC motility is reliant on NO production, attempts could also be made to eliminate NO from the cell culture using NOS inhibitors to examine its effect on migration. The effects of antioxidants to oppose these negative effects could also be tested.

Previous work by other groups also merits consideration. A large proportion of published work regarding the negative influence of oxidative stress on EC migration has surrounded oxLDL, which acts as a key oxidant *in vivo*. Overwhelming evidence exists to support the role of oxLDL in atherosclerosis (232) and the triggering of acute coronary syndromes (233), therefore it is not surprising that EC function is a target for its pathological actions. Given that repair of injured blood vessels is dependent on EC migration following arterial injury, the effect of oxLDL on this phenomenon is likely to be important after PCI. OxLDL increases intracellular ROS production by ECs (234). Murugesan *et al.* (235) found that oxLDL markedly reduced EC migration using a razor wound assay and this effect was concentration- and oxidation-dependent. In this work, the antimigratory activity of oxLDL was not reliant on mortal cell injury, since migration was re-established after removal of oxLDL from the culture. Chavakis *et al.* (236) also reported that oxLDL inhibited VEGF-induced EC migration, without inducing apoptosis or necrosis. This observation was associated with dephosphorylation of the serine/threonine kinase Akt/protein kinase B, which led

to deactivation of eNOS and a reduction in NO generation, thus inhibiting a process which is partly NO dependent (237).

More evidence that ROS are pivotal in inhibiting EC migration by oxLDL has been provided by van Aalst *et al.* (126), and this work also provides an important insight into my results. As in other studies, the antimigratory effects of oxLDL on ECs was demonstrated using the razor scrape assay. Inhibition of EC migration was also confirmed using other agents known to stimulate ROS production (lysophosphatidylcholine and naphthoquinones). In this study, the effect of oxLDL was blocked by the presence of SOD or NADPH oxidase inhibitors (DPI, quinacrine and hydralazine). It was not affected by mannitol or glutathione, which inactivate OH^- anions, or catalase, which scavenges H_2O_2. These data suggest that oxLDL inhibits migration of ECs by causing the release of O_2^-, produced enzymatically by NADPH oxidase. This, in turn, supports the proposal that the antimigratory effects of X/XO on ECs observed in my study may have been mediated by O_2^-.

Interestingly, direct administration of H_2O_2 (10-50 µM) had no effect on EC migration, however higher concentrations (> 100 µM) caused ECs to lift off the plate (126). This probably explains the general cytotoxicity of the X/XO reaction in my study, which is known to produce large quantities of H_2O_2 (238). The inability of H_2O_2 to inhibit EC migration suggests that different ROS may lead to cell death compared to those that modulate motility. $ONOO^-$, which is created by the rapid reaction between NO and O_2^-, has been shown to impair actin polymerisation and inhibit movement of neutrophils and fibroblasts (239;240),

therefore it could also affect ECs. However, the importance of this potent oxidising substance in my study, or that of others, with regard to EC migration has not been established.

3.4.3 Effect of probucol on EC migration

I found that 20 µM probucol had no effect on EC migration in this assay. Moreover, I have shown that probucol at the same concentration was unable to preserve EC migration in the presence of X/XO. This was interesting, since some data already exists to support the use of probucol to encourage endothelial healing in animal models (data from Chapter 1, Table 1.1). Pre-incubation with probucol also reportedly provided ECs with protection against fatal oxidative injury (129). However, the inability of probucol to preserve EC migration in the presence of oxidative stress is in agreement with another study which utilised oxLDL as a source of ROS (127). In an identical fashion to my results, probucol led to minimal improvement in EC migration compared with oxidative stress. I propose that probucol is insufficient to oppose the inhibitory effects of excessive ROS on EC migration, possibly due to its delayed uptake into ECs (163;241) and poor solubility in aqueous solution.

3.4.4 Limitations

Significant limitations exist in this work and the presented data should be interpreted in this context. In a few cases, the razor scrape method caused marked damage to the EC monolayer, resulting in an irregular starting line and rendering some wells invalid for the EC migration assay. The severe generalised toxicity of X/XO severely limited the number of valid experiments, therefore the

number of different cell populations available for each group was low and only a single concentration of probucol was tested. The concentration of probucol chosen was however similar to that used in previous studies (127). Nevertheless, a concentration-response curve for probucol would have been valuable. It would have been interesting to confirm the nature and extent of general cell toxicity of X/XO using specific methods such as lactate dehydrogenase release assay or trypan blue staining (242). Confirmation that EC migration was abolished in the absence of cell death could have been tested by reseeding the ECs in standard growth medium to ensure their motility was re-established. However, even in those groups in which migration virtually ceased completely, cells maintained their normal appearance and adherence in contrast to the findings in chapter 5 (Figure 5.2.1). Direct measurement of O_2^- production in the EC culture medium would have been valuable to quantify and characterise ROS generation by the X/XO reaction and the effect of probucol, although the former could be indirectly confirmed by chemiluminometry reported in Chapter 2.3.2. The observer performing microscopic examination of EC migration was not blinded, similar to previous work published in this area (126;127). The standard error within each group was relatively low, suggesting that the selection of analytical fields did not suffer from major variability.

3.4.5 Conclusions

Taken together, the results of this study and others indicate that oxidative stress is an important modulator of EC migration. The fact that ROS may inhibit EC migration has direct clinical relevance. In the setting of PCI, potential sources of ROS include oxLDL already present in atherosclerotic lesions and the

consequence of the procedure itself, which is known to generate ROS (243). Elevated levels of circulating and local ROS, especially O_2^-, could contribute to delayed stent healing after PCI. Retarded re-endothelialisation would be expected to encourage SMC proliferation and exacerbate neointimal thickening. Successful methods to reduce local levels of ROS, particularly O_2^-, may promote endothelial recovery and reduce ISR. Whilst this study provides no evidence that probucol is beneficial, the evaluation of more potent antioxidants with improved pharmacokinetic profiles to achieve this goal may present an important step towards the development of novel effective DES.

Chapter 4

An *in vitro* evaluation of nitrone D

as a potential novel DES coating

4.1 Introduction

Nitrone-based antioxidants represent a family of electron paramagnetic resonance (EPR) spin-trapping agents, developed to trap ROS within biological tissues (244). Investigation of several novel nitrone-based compounds in human fibroblasts has suggested that the analogue, nitrone D, may possess the most favourable biological profile to take forward into preclinical assessment (245). The chemical structure of nitrone D is shown in Figure 4.1. Nitrone D is a highly lipophilic molecule which embeds deeply within biological membranes. This renders it potentially appealing as a therapeutic agent in atherosclerosis and other inflammatory conditions characterised by lipid peroxidation and heightened oxidative stress. Nitrone D has been shown to be a functionally effective antioxidant by providing protection to human fibroblasts against oxidative challenge with 150 μM H_2O_2 (245). Another important effect of nitrone D was the five-fold increase in relative SIRT 1 expression (245). SIRT 1 is a deacetylase protein which is implicated in a broad range of cellular functions (246;247). Importantly, SIRT 1 promotes EC proliferation and survival (248). Furthermore, upregulation of SIRT 1 appears to prevent oxidative stress-induced EC senescence, which may help to maintain a functional endothelium (249). Established DES coatings (sirolimus and everolimus) decrease SIRT 1 expression and cause a senescent phenotype in ECs, an effect which was reversed by SIRT 1 overexpression (250).

Bearing in mind these encouraging data, nitrone D was chosen as a potentially suitable DES coating for evaluation. First, I assessed the effect of nitrone D on

WBA to determine if it had favourable antithrombotic properties that would be attractive as a DES coating. As no data exists for the effects of nitrone-based antioxidants on platelet or blood aggregation, this would provide important safety data for a novel compound to be potentially used as a stent coating. If antithrombotic properties were identified, that may potentially inhibit early stent thrombosis and to some extent neointimal formation, although the antirestenotic efficacy of anticoagulant coated stents has been limited to date (251;252). Second, and more importantly, I set out to test the effect of nitrone D on migration of cultured ECs. If nitrone D were to display favourable characteristics *in vitro*, then it would be investigated as a DES coating in a preclinical *in vivo* model to assess whether it would improve endothelial regrowth, reduce ISR or lower the risk of stent thrombosis.

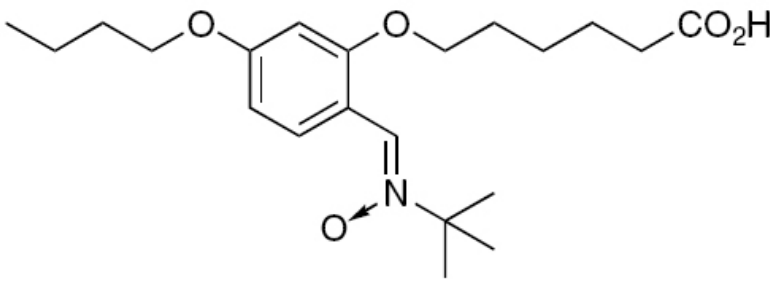

Figure 4.1 The chemical structure of nitrone D.

4.2 Methods

4.2.1 Chemiluminescence

Lucigenin chemiluminescence was performed with nitrone D to establish that it was acting as an antioxidant, according to the method in Chapter 2.2.1. Antioxidant efficacy was assessed using 50 U/ml SOD as a positive control in the presence of 100 μM X and 10 mU/ml XO.

4.2.2 Study population for WBA

This study was approved by the West Glasgow and University of Strathclyde ethics committees. All participants were provided with a PIS and gave their informed written consent. All patients were recruited from adult patients with angiographic evidence of stable CHD, who attended the Western Infirmary Glasgow for out-patient cardiac appointments. All patients were over age 18 and receiving chronic oral aspirin therapy. Patients were excluded if there was a history of myocardial infarction within previous 3 months, they were unable to give informed consent or if they were taking any other antithrombotic therapy (as in Chapter 2).

4.2.3 Whole blood aggregometry

The method of WBA is described in Chapter 2.2.3. To assess the effect of nitrone D, blood was incubated with increasing concentrations of nitrone D (1-100 μM), for 1 minute prior to addition of each agonist. Finally, to assess the effect of nitrone D in the presence of oxidative stress, 100 μM nitrone D was given prior to adding 100 μM X and 10 mU/ml XO, 1 minute before the addition of agonist.

4.2.4 EC culture and migration assay

The methods of EC culture and EC migration assay are described in Chapter 3.2.1-3.2.2. EC migration after 24 hours was measured under control conditions and after exposure to 100 µM nitrone D. Control cells were treated with the relevant vehicle (PBS).

4.2.5 Statistical analysis

All data are expressed as the mean ± SEM, unless otherwise stated. For WBA, individual aggregation values were calculated as a percentage of aggregation compared to control conditions. For EC migration, the number of ECs that had migrated across the 1,300 µm starting line after 24 hours was counted. All EC migration experiments were performed in duplicate, on cultures derived from at least 2 different animals. Groups were compared using a t-test or repeated measures analysis of variance with post-hoc Dunnett's test. Statistical significance was confirmed at $p < 0.05$. Statistical analysis was performed using the SPSS statistical software package 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

4.3 Results

4.3.1 Confirmation of antioxidant activity of nitrone D

The addition of 100 µM nitrone D prior to the reaction between 100 µM X and 10 mU/ml XO abolished chemiluminescence in a similar fashion to 50 U/ml SOD, indicating that nitrone D was acting as an effective antioxidant (Figure 4.2).

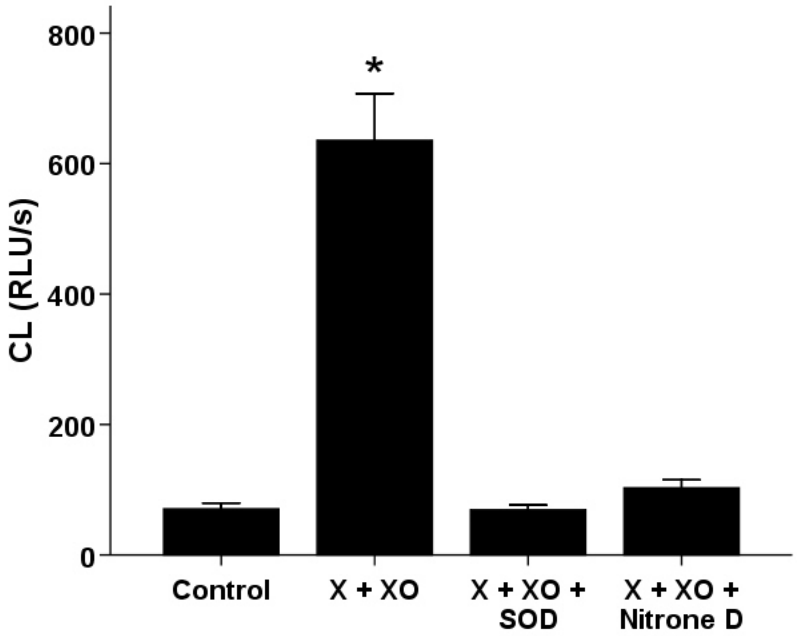

Figure 4.2 The effect of nitrone D on X/XO-induced chemiluminescence. CL due to 100 μM X and 10 mU/ml XO with the effect of 50 U/ml SOD and 100 μM nitrone D (n = 4), * $p < 0.001$ vs. control.

4.3.2 Baseline characteristics of WBA patients

The baseline characteristics of 10 patients included for WBA are shown in Table 4.1. The prevalence of additional cardiovascular risk factors was high and most patients were taking standard drug therapy for CHD. As in Chapter 2, all patients were taking regular aspirin and no patient was taking clopidogrel or any other antithrombotic medication.

Table 4.1 Baseline characteristics of WBA patients

	Prevalence, (n = 10)
Patients' characteristics	
Age, mean (SD), y	66.9 (10.0)
Male, n (%)	9 (90.0)
Current smoker, n (%)	3 (30.0)
Hypertension, n (%)	4 (40.0)
Hypercholesterolaemia, n (%)	5 (50.0)
Diabetes mellitus, n (%)	5 (50.0)
Family history of premature CHD, n (%)	3 (30.0)
Previous myocardial infarction, n (%)	5 (50.0)
Previous stroke, n (%)	0 (0.0)
Previous PCI or CABG, n (%)	1 (10.0)
Multivessel disease, n (%)	6 (60.0)
Preserved LV function, n (%)	8 (80.0)
Heart failure, n (%)	2 (20.0)
Drug treatment	
Aspirin, n (%)	10 (100.0)
Clopidogrel, n (%)	0 (0.0)
Statin, n (%)	8 (80.0)
ACE inhibitor or ARB, n (%)	6 (60.0)
Beta-blocker, n (%)	8 (80.0)
Calcium channel blocker, n (%)	4 (40.0)
Diuretic, n (%)	6 (60.0)
Nitrate, n (%)	6 (60.0)
Nicorandil, n (%)	5 (50.0)

4.3.3 Effect of nitrone D on WBA

The effect of nitrone D on WBA in CHD patients is shown in Figures 4.3 and 4.4. Nitrone D caused a concentration-dependent inhibition of WBA compared to control, for both agonists. Although the effect of each individual dose fell short of statistical significance, a linear trend was evident for each agonist (both $p < 0.05$ for trend). As in Chapter 2, 100 µM X and 10 mU/ml XO inhibited WBA non-significantly compared to control. However, in the presence of 100 µM X and 10 mU/ml XO, 100 µM nitrone D inhibited WBA significantly in response to collagen by 18.4% (95% CI 3.9%-33.0%, $p < 0.05$) and in response to ADP by 39.0% (95% CI 0.2%-77.8%, $p < 0.05$).

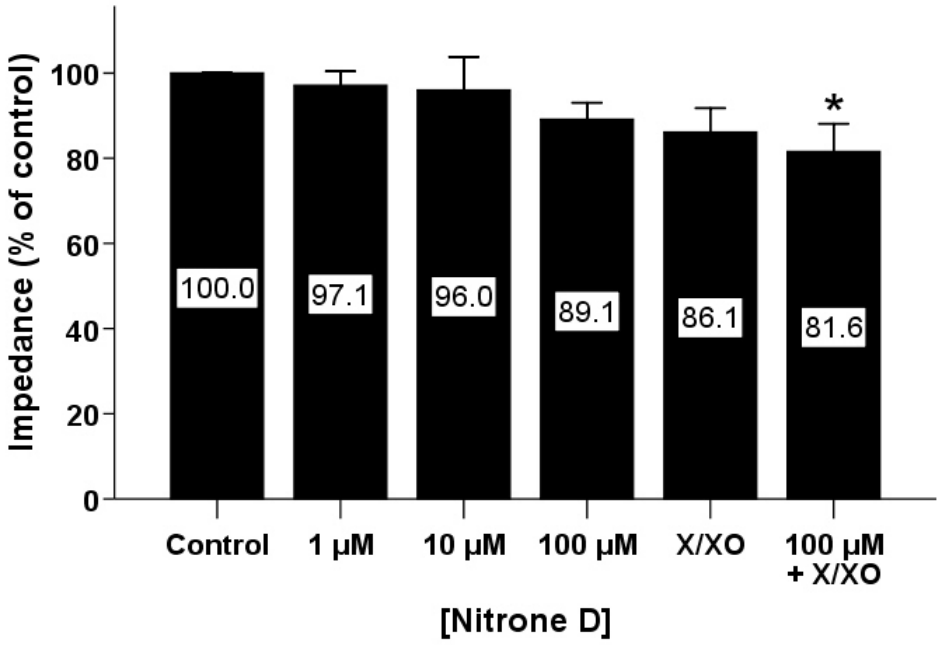

Figure 4.3 The effect of nitrone D on WBA in response to collagen. Nitrone D (1-100 µM) caused a concentration-dependent inhibition of WBA in response to 3 µg/ml collagen in CHD patients ($p < 0.05$ for trend). 100 µM nitrone D and X/XO significantly inhibited WBA ($n = 9$), * $p < 0.05$ vs. control.

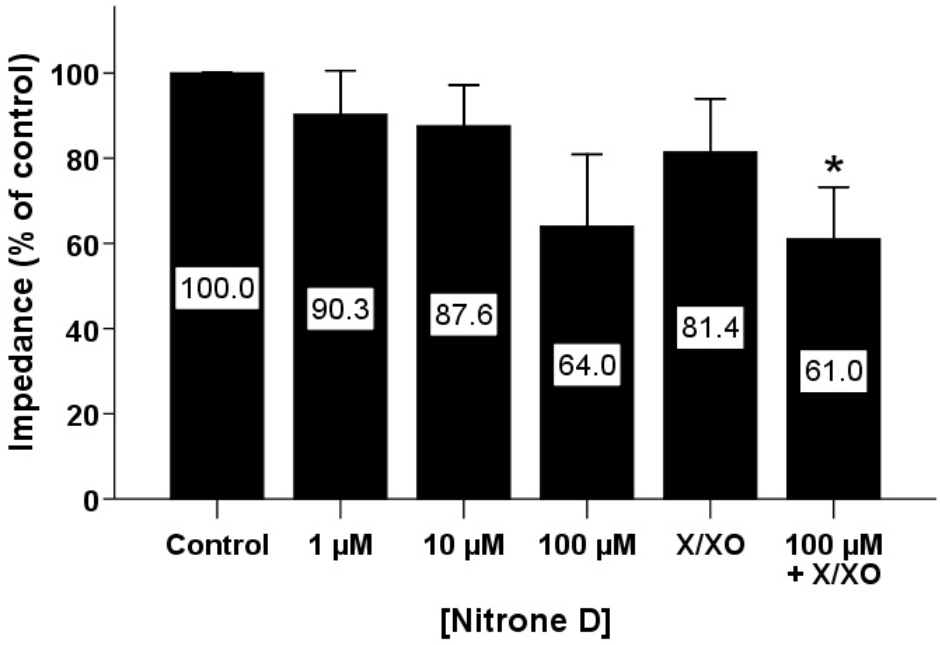

Figure 4.4 The effect of nitrone D on WBA in response to ADP. Nitrone D (1-100 µM) caused a concentration-dependent inhibition of WBA in response to 5 µM ADP in CHD patients ($p < 0.05$ for trend). 100 µM nitrone D and X/XO significantly inhibited WBA ($n = 7$), * $p < 0.05$ vs. control.

4.3.4 Effect of nitrone D on EC migration

Initially, the effect of nitrone D alone on EC migration was studied (Figure 4.5). 100 µM nitrone D caused a marked reduction in EC migration compared with control (1.1 ± 0.5 vs. 6.4 ± 1.8 migrating cells/wound, respectively [$p < 0.01$]). Given that nitrone D alone severely limited EC migration, its influence on the migration of X/XO-treated cells was not evaluated.

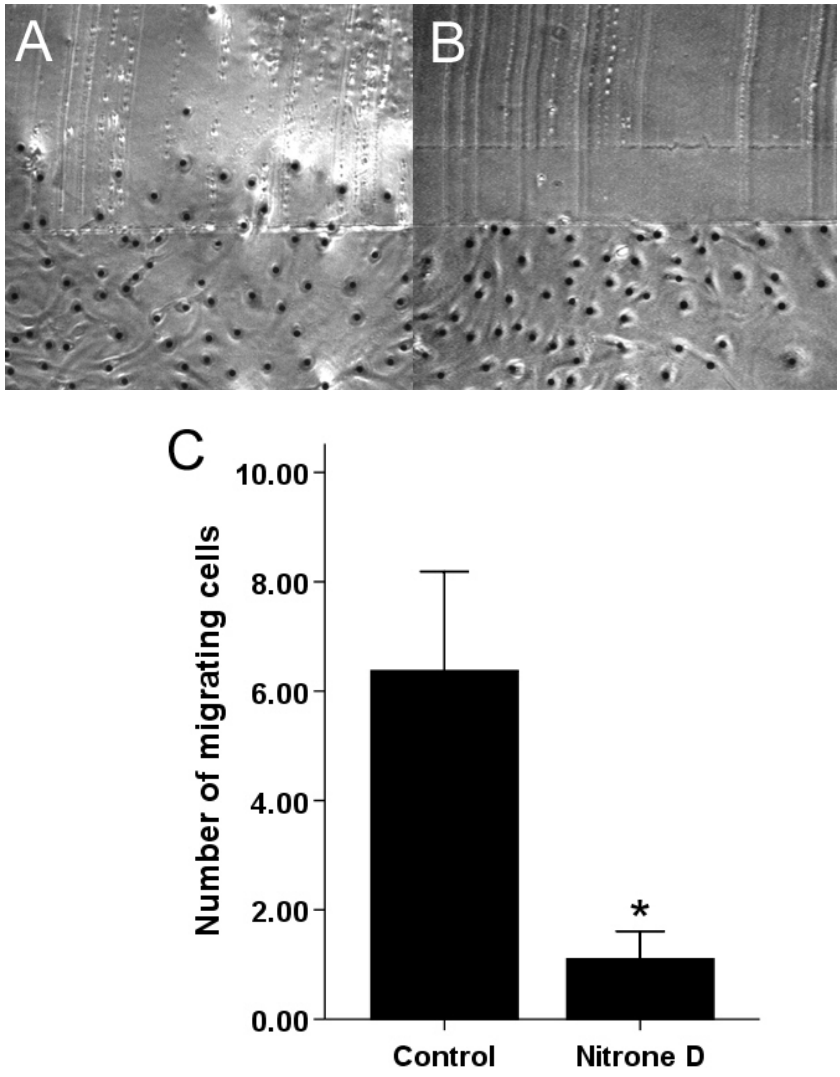

Figure 4.5 The effect of nitrone D on EC migration. Representative photomicrographs of vehicle (A) and 100 µM nitrone D (B) in the scratch wound assay. C, 100 µM nitrone D severely inhibited EC migration compared with control (n = 4), $p < 0.05$ vs. control.

4.4 Discussion

4.4.1 Nitrone D as an antioxidant

Nitrone D was found to perform well as an antioxidant in chemiluminescence experiments. In the presence of X/XO, nitrone D was able to quench O_2^- from the surrounding environment in a similar fashion to SOD.

4.4.2 Effect of nitrone D on WBA

Although no individual concentration of nitrone D was found to inhibit WBA, there was a trend across the concentration range of an inhibitory effect, which may have reflected increasing antioxidant activity. Although antioxidants may suppress the pro-aggregatory effects of platelet-derived O_2^- (177), this effect was not confirmed by this study. When local oxidative stress was enhanced by X/XO, addition of 100 µM nitrone D led to a statistically significant reduction in WBA. As discussed in Chapter 2, the overall effect of exogenous ROS generated by X/XO in this assay is inhibitory, probably due to the relative over-production of anti-aggregatory ROS such as H_2O_2 and $ONOO^-$, rather than O_2^-. I speculate that nitrone D may have been less protective against inhibitory ROS; thus, co-administration of X/XO and nitrone D resulted in additive inhibitory effects on WBA, which became statistically significant. I have found no data to suggest that elevation of SIRT 1 or reduction in p16 and p21 gene expression (245) are alternative potential mechanisms of platelet inhibition, although this was unlikely given the time frame of the experiments. To my knowledge, the effect of nitrone D on platelet function has not previously been described. Therefore, this work

provided evidence that nitrone D is likely to have, at most, a minor inhibitory effect on WBA.

4.4.3 Effect of nitrone D on EC migration

Surprisingly, nitrone D (100 µM) caused a marked reduction in EC migration. In fact, migratory activity of ECs was virtually abolished by nitrone D. This was an unexpected finding and rendered nitrone D very unattractive as a novel DES coating, which would ideally accelerate endothelial regeneration. Although only a single concentration of nitrone D was tested, I speculate that concentrations less than 100 µM would not have been effective overall, since nitrone D was not toxic to human fibroblasts up to 300 µM and relatively high concentrations are desirable to trap ROS and the carbon-centred radicals resulting from them (245). Human fibroblasts grown in nitrone D had elevated SIRT 1 transcription, consistent with these cells being more adapted to growth and survival (245). Therefore positive effects were anticipated in this study, as SIRT 1 is emerging as a key protective regulator of ECs against oxidative stress (253;254). The reason(s) for the marked inhibitory effect of nitrone D on EC migration is unclear, but can be explored by considering the processes required for cell motility.

Migration of ECs involves cytoskeleton reorganisation, extension of lamellipodia and filopodia, with formation and detachment of adhesions at the edges of the cell to facilitate movement. Nitrone D is known to reduce p21 expression in human fibroblasts (245) and this effect may be relevant to EC migration because previous data supports a role for p21 activated kinases in the coordination of

leading edge adhesion formation and contraction/detachment of the trailing edge of the cell (255). Another strong possibility was that the potent antioxidant properties of nitrone D were responsible for reduced EC migration. It is now recognised that ROS have a dual role in affecting cell migration, which can be stimulatory or inhibitory, depending on the type, amount and duration of exposure. Compelling studies by Moldovan *et al.* (256) and Ikeda *et el.* (257) found that ECs at the margins of a wound assay produce small amounts of ROS, which appear to be essential for normal migration. Normal cell motility in animals is based on changes in the actin cytoskeleton, which depends on the response to numerous signals (258). Actin polymerisation, necessary for cytoskeleton reorganisation, is mediated by targeted physiological release of O_2^- from ECs (256). Therefore, potent antioxidants acting deep within cells, such as nitrone D, could disturb this process and lead to impaired cell motility. In support of this, a SOD mimetic and inhibitor of NADPH oxidase both limited EC migration (256). A finely balanced redox state is likely to support EC movement which, like platelet aggregation, is reliant on physiological cellular ROS generation, but may also be disrupted by disproportionately high levels of oxidative stress present in pathological states.

4.4.4 Limitations

It would have been beneficial to test a variety of concentrations and construct a concentration-response curve for nitrone D in the EC migration assay. The effect of X/XO caused a lower chemiluminescence signal compared to the values in Chapter 2. This appeared to be due to considerable daily variability in the assay, which was unexplained. As a result, to maintain scientific validity, all

experiments for a given section were performed in succession on the same day and excellent reproducibility was achieved for identical conditions within each group. As in previous chapters, the relevance of *in vitro* assays in whole blood to the clinical setting is limited. Recent work has shown that some methods of *in vitro* platelet testing can predict clinical events after PCI and although supportive data is not available for WBA, this method correlates well with light transmission aggregometry (259), which does display some prognostic value (260).

4.4.5 Conclusions

Although I found that nitrone D displayed attractive properties by weakly inhibiting WBA, it caused a marked reduction in EC migration. This latter characteristic is highly undesirable as a potential DES coating because, if occurring *in vivo*, it could significantly impair endothelial healing. Given that promotion of re-endothelialisation was a primary objective of this research project, nitrone D was no longer considered suitable as a potential novel antioxidant DES coating.

Chapter 5

The effects of a succinobucol-eluting stent in a porcine coronary model

5.1 Introduction

The advent of DES has enabled a vast array of compounds to be tested as local treatments after stent deployment in a bid to modify arterial healing. The primary objective of early DES was to inhibit neointimal hyperplasia and resulting ISR, the main drawback of BMS. This goal has largely been achieved by the first generation of DES, now in widespread clinical use. However, whilst DES have successfully decreased the incidence of ISR, the polymers and drugs used as coatings can cause delayed arterial healing and inflammation, which both increase the risk of late stent thrombosis, a potentially life-threatening complication (261). Thus, much effort is now being directed towards the development of novel polymer-free DES using drugs which inhibit ISR, but also promote healing and reduce inflammation.

Previous studies have shown that probucol can reduce ISR by inhibiting neointimal hyperplasia and improving re-endothelialisation after stent injury (150;155). Probucol is thought to mediate these beneficial effects via its antioxidant properties (148;262) and up-regulation of heme oxygenase-1, which induces vascular SMC apoptosis and promotes EC function (160). Unfortunately, in clinical trials, the effects of oral probucol have been less consistent (153;154;156;157). The efficacy of probucol as a DES coating has been evaluated in a preclinical model (263) and subsequently in the ISAR-TEST-2 clinical trial, which showed that a non-polymer based dual probucol/rapamycin-eluting stent was equivalent to a polymer-based rapamycin-eluting stent (264). This was remarkable, given that rapamycin is known to have superior effects if

delivered using a polymer (265). It is not clear whether probucol was exerting polymer-like properties or if it was directly beneficial to vascular healing. The efficacy of a probucol-eluting stent is currently unproven.

Succinobucol is a novel compound designed to improve upon many of the chemical and pharmacological properties of probucol (Figure 5.1). It has several properties highly attractive as a novel DES coating. The introduction of a monosuccinate moiety onto a phenol group renders succinobucol slightly more hydrophilic than probucol. This leads to considerably enhanced cellular uptake compared with probucol, which suffers from poor bioavailability and requires preloading for several weeks (157;163). Succinobucol potently inhibits basal and H_2O_2-induced levels of ROS released from human ECs and monocytic cells in a concentration-dependent fashion, whereas probucol has a much weaker effect (163;166). Succinobucol also exerts antiproliferative effects on vascular SMCs, with 50% inhibition at 5 µM (165;166), compared with probucol which was shown to exert no significant effect on SMC proliferation up to 100 µM (165). Succinobucol also has important anti-inflammatory actions. In previous studies, succinobucol potently inhibited pro-inflammatory cytokine release by monocytes and the expression of several pro-inflammatory adhesion molecules, including vascular cell adhesion molecule-1 and MCP-1 (163-166), which may contribute significantly to neointimal development (266;267). The anti-inflammatory effects of probucol in these studies was greatly limited. These comparisons with probucol may partly explain the poor results of oral probucol in clinical studies (154;157) and the negative preclinical effects of a probucol-eluting stent (158).

Finally, succinobucol also reduces platelet aggregation, which may be beneficial in lowering the risk of early stent thrombosis (268).

In the ARISE (Aggressive Reduction of Inflammation Stops Events) clinical trial, oral succinobucol reduced the incidence of myocardial infarction, stroke and diabetes mellitus (269). However, succinobucol caused more adverse effects than placebo, worsened lipid profiles and increased the incidence of atrial fibrillation. CART-1 tested oral succinobucol following coronary stenting, but the reduction in ISR was significant only if drug compliant patients were analysed separately (156).

Succinobucol has never been evaluated as a DES coating and appears to offer significant advantages over probucol. It is possible that locally targeted therapy using stent-based delivery of succinobucol could improve efficacy and reduce systemic adverse effects. On the other hand, if negative cellular actions outweigh the beneficial effects of succinobucol, delivery of high concentrations into the artery wall may result in localised toxicity. The combination of succinobucol with a recognised inhibitor of ISR such as rapamycin may potentiate beneficial effects on neointimal formation and negate the proinflammatory effects of rapamycin on injured arterial tissue (170). There is also some evidence suggesting that oxidative stress may be linked to rapamycin resistance (270), therefore local application of an antioxidant may improve the efficacy of rapamycin. Finally, succinobucol may improve the elution profile of rapamycin in the absence of a polymer, by retarding drug release in a similar fashion to probucol (91) leading to improved efficacy. The aim of this study was to test

whether succinobucol alone, or in combination with rapamycin, coated on a polymer-free DES would have a favourable effect on ISR and vascular healing after stent implantation.

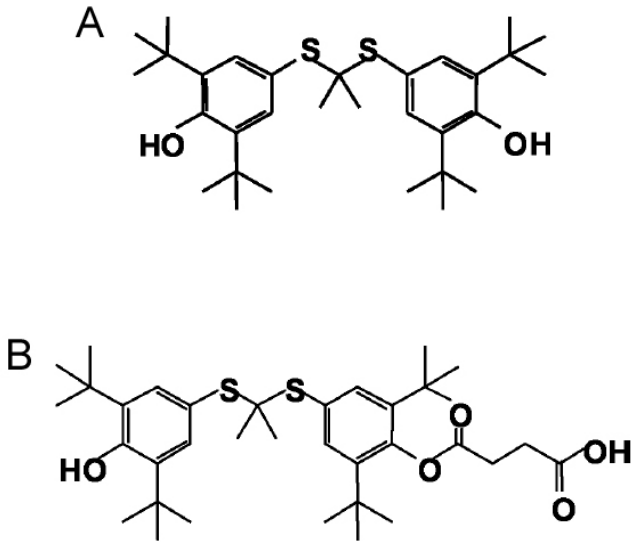

Figure 5.1 The chemical structure of probucol (A) and succinobucol (B).

5.2 Methods

5.2.1 Drugs

Succinobucol ([butanedioic acid, mono[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenyl]ester], previously known as AGI-1067), the monosuccinic acid ester of probucol, was synthesized by esterification of probucol (Sigma-Aldrich, Poole, Dorset). The identity of succinobucol was confirmed by NMR spectroscopy and purity was in excess of 99%. Succinobucol is metabolically stable and no significant active metabolites are formed *in vivo* (271). Rapamycin

(sirolimus) is a macrocyclic triene antibiotic with potent antiproliferative, anti-inflammatory and immunosuppressive effects. It forms a complex with FKBP12, which subsequently binds to and inhibits the molecular target of rapamycin (mTOR), causing arrest of cell proliferation. Rapamycin (purity ≥ 95%) was purchased from Cfm Oskar Tropitzsch (Marktredwitz, Germany).

5.2.2 DES coating

The Yukon® DES (Translumina, Hechingen, Germany) used in this study consisted of a pre-mounted, sandblasted 316L stainless steel microporous stent, which is designed for individualised and dose-adjustable on-site stent coating. The microporous stent surface enables increased drug deposition and improves the drug reservoir capacity, allowing for retarded drug release without recourse to a polymer (272). All stents were 3.5 mm diameter and 16 mm length. The BMS were uncoated versions of the Yukon® stent. All stent coating solutions consisted of drug(s) dissolved in 99.5% ethanol. Sterile stent cartridges were placed in the coating device (Figure 5.2) and connected to a 1 ml syringe which contained a predetermined volume of coating solution (0.33 ml for a 16 mm stent). Stent coating was achieved by a mobile ring containing 3 jet units, which allowed uniform spraying of drug solution onto the stent surface. The total time to spray coat the stent was approximately 6 minutes. The coating device dried the stent surface automatically by evaporating the ethanol with pressurised air (4-6 atmospheres). When spray coating was complete, the stent cartridge was removed from the machine and set aside for use within 4 hours. In the absence of prior data, three succinobucol coating solutions: 0.5% (5 mg/ml), 1% (10 mg/ml) and 2% (20 mg/ml) were tested. The succinobucol concentration that produced

optimal strut coverage, using scanning electron microscopy (Hitachi S-4800), was selected for preclinical evaluation. The concentration of rapamycin coating solution (2%) was derived from favourable data using an identical Yukon® stent platform (272;273). Three DES were evaluated: a succinobucol-eluting stent (SES); a rapamycin-eluting stent (RES); a dual succinobucol/rapamycin-eluting stent (SRES) and these were compared to an uncoated Yukon® stent (BMS).

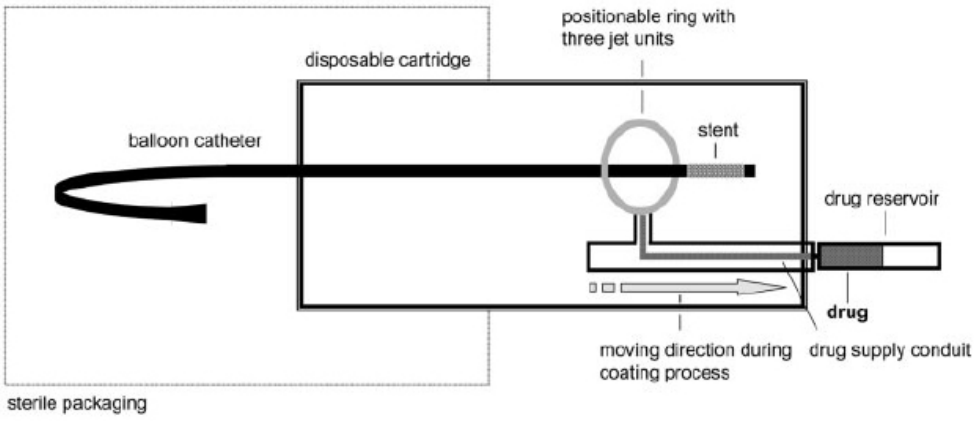

Figure 5.2 The DES coating device. Individualised, dose adjustable DES coating was achievable using a predetermined volume of coating solution which was sprayed via three mobile jet units onto the microporous stent surface under sterile conditions.

5.2.3 Preoperative care and anaesthesia

Male Large-White/Landrace pigs (approximately 10 weeks old, 20-25 kg) were allowed to acclimatise for at least a week before commencement of the study. Each pig was preloaded with oral aspirin 300 mg and oral clopidogrel 300 mg 3 days prior to the procedure, followed by normal diet with supplementation of oral aspirin 75 mg and oral clopidogrel 75 mg daily for the remainder of the study. On the morning of the procedure, 2-3 stents were prepared for implantation. The pig was sedated by an intragluteal injection of

tiletamine/zolazepam (Zoletil®) 100 mg i.m. and a mixture of inhaled isoflurane (1–2%) in oxygen/nitrous oxide. Intravenous access was obtained via cannulation of the marginal ear vein and propofol (Rapinovet®) 30 mg i.v. was administered slowly over 1-2 minutes. Immediately, tracheal intubation was performed using a 5.5 French veterinary tracheal tube. The tracheal tube was connected to the ventilator and general anaesthesia maintained throughout the procedure using a mixture of isoflurane (1–2%) in oxygen/nitrous oxide. Pigs were given buprenorphine (Vetergesic®) 0.15 mg i.m. for perioperative analgesia and ampicillin (Amfipen®) 350 mg i.m. as antibiotic cover. Electrocardiography leads were connected to three limbs for cardiac monitoring.

5.2.4 *In vivo* stenting

Heparinised saline (5,000 units heparin in 500 ml 0.9% saline) was used to flush all catheterisation equipment. The left groin area of the pig was sterilised using povidone-iodine (Betadine®) solution and the surgical field was prepared with sterile drapes. A 5 cm incision was made with a sterile scalpel and the subcutaneous tissue was divided. The underlying muscles were separated by blunt dissection to expose the left femoral neurovascular bundle. The left femoral artery was dissected carefully to separate it from the femoral vein and femoral nerve, which allowed a blunt suture holder to pass two absorbable sutures (Dexon™ II) around the femoral artery (Figure 5.3A). The femoral artery was accessed between the two sutures using a human transradial artery access kit (Arrow® International UK Ltd, Middlesex). Arterial puncture was confirmed by pulsatile arterial flow. The 0.025 inch soft tipped guidewire was passed through the needle into the arterial lumen, the needle was removed and a 6 French sheath

and dilator were placed over the wire. The wire and dilator were removed and the sheath was flushed with heparinised saline (Figure 5.3B).

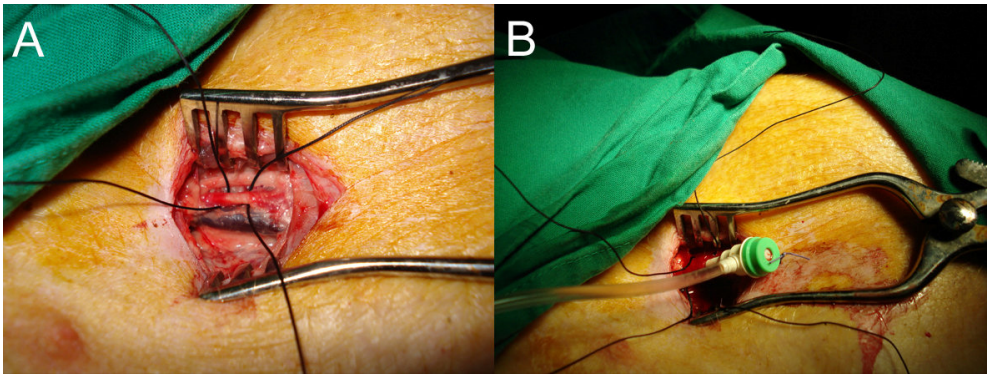

Figure 5.3 Intra-arterial access via the left femoral artery in the pig. A, Two sutures were placed around the femoral artery, which lies between the femoral vein and femoral nerve. B, The sheath was inserted after arterial puncture and wire access.

Heparin 2000 units (100 units/kg) i.a. was administered via the sheath. Real time fluoroscopy (Siremobil 4N, Siemens) was used to image the catheterisation. A 0.035 inch J-tipped guidewire was inserted into a 6 French coronary guiding catheter and both were advanced to the aortic arch. The guidewire was removed and the catheter was attached to a 3 port manifold, connected to an electronic pressure transducer, heparinised saline flush and radiographic contrast (Omnipaque 140, GE Healthcare), with a luer lock 10 ml syringe (BD 300-912) attached to the distal end for intra-arterial injection. The catheter tip was placed at the ostium of the left or right coronary artery, confirmed by contrast injection. Suitable guiding catheters were Judkins Right 3.5 or 3 for both coronary arteries or Judkins Left 3.5 or Amplatz Right 1 for difficult intubations. A 0.014 inch coronary guidewire was introduced and advanced to a distal portion of a major coronary artery. A Yukon® stent (type of stent was blinded to the operator) was

prepared by passively filling the stent balloon with 1:1 contrast:saline and advanced over the guidewire into a stable position in the proximal or mid coronary artery, avoiding major coronary branches. Balloon inflation was performed for 20 seconds, aiming for a stent:artery ratio of 1.1-1.2:1 (typically 9-12 atmospheres), i.e. slight over-sizing. Appropriate injury was confirmed by angiography before the balloon and guidewire were removed (Figure 5.4).

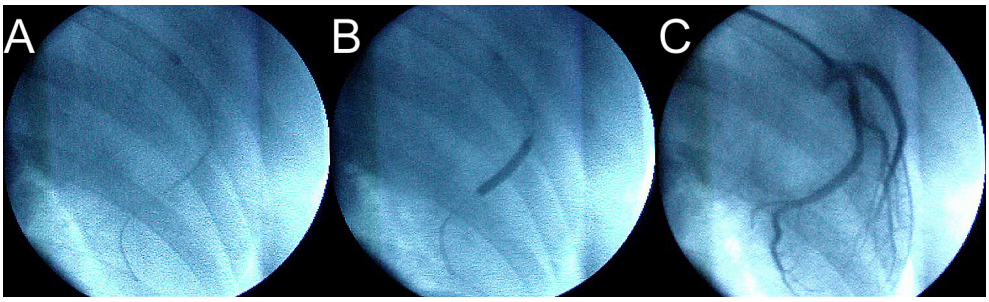

Figure 5.4 Angiograms of coronary stent implantation in the pig. A, Catheter in left main coronary artery and guidewire in distal left circumflex coronary artery. B, Inflation of balloon and stent deployment. C, Final angiographic result with slight oversizing of stent:artery ratio. All angiograms shown are right anterior oblique 30º projection. In this view, the left circumflex is medial to the left anterior descending coronary artery.

This procedure was repeated in a total of 2-3 coronary arteries in order to implant 2-3 stents per pig. Succinobucol was undetectable in blood samples 1 hour and 1 day after stenting therefore implantation of multiple stent types in each animal was permitted in accordance with published guidelines (175). During the procedure, the pig received an intravenous saline infusion (approximately 250 ml) to support blood pressure. The catheter and sheath were removed and haemostasis was achieved by manual pressure (10-20 minutes) or ligation of the femoral artery, if ongoing bleeding. The subcutaneous tissues were closed with

absorbable sutures (Dexon™ II) and the skin was closed with non-absorbable sutures (Ethilon®). Anaesthesia was terminated and the pig was extubated. Adequate recovery was ensured and the pig returned to the pen. All premature and unexpected deaths were examined by post-mortem, gross evaluation and stent examination. Approval was granted by Strathclyde University Ethics Review Committee and all procedures were performed under Home Office Project Licence number 60/3410.

5.2.5 Pharmacokinetic studies

Drug loading of succinobucol was quantified by *in vitro* elution of the SES in pure ethanol, followed by HPLC analysis. To determine *in vivo* release characteristics of the SES, stents were harvested 1 hour, 1, 3, 7, 14 and 28 days after implantation. At the end of each predefined period, the pig was sedated and euthanized by a lethal dose of sodium pentobarbital (Euthatal®) 3000 mg i.v. The heart was removed via midline thoracotomy and placed in saline. The coronary arteries containing stents were carefully dissected from connective tissue. Stents were excised and surrounding arterial tissue was homogenised. Succinobucol in surrounding arterial wall and remaining on the stent was extracted into acetonitrile. Samples were chromatographed on a Sphereclone ODS (2) column (5 µm particle size, 150 × 4.6 mm [Phenomenex, UK]). Samples were injected using an autosampler and pump system (Gynotek 480) in 20 µl aliquots, at a mobile phase flow rate of 1 ml/min acetonitrile:water (92.5:7.5). The detector (Detector 432, Kontron Instruments, UK) output was measured at a wavelength of 242 nm.

5.2.6 Tissue fixation and processing

To determine efficacy, stents were harvested 28 days after stent implantation. At the end of the study period, each pig was sedated and euthanized as previously described. The heart was examined for gross evidence of myocardial infarction. The coronary arteries were dissected free from connective tissue and the stented segments were excised, including 3-4 mm of non-stented artery at each end. The segments were rinsed with saline to remove non-adherent clot. The specimens were fixed overnight in 10% formal saline, in an Eppendorf tube at 4 ºC, followed by dehydration in 100% acetone, overnight at 4 ºC. The specimens were then placed in glycol methacrylate (Technovit® 8100, Kulzer) infiltrating solution in an Eppendorf tube and left overnight at 4 ºC. The following day the specimens were removed and placed in a small plastic cylindrical tube, using circumferential foam padding to ensure correct orientation (distal end of the stent at the base of the tube). The tube was filled with Technovit® 8100 embedding solution and air bubbles were removed using forceps, before placing in the fridge to allow the plastic resin to harden. The Technovit® 8100 infiltrating and embedding solutions were made up according to the manufacturer's instructions. This produced a small cylindrical block of plastic resin containing the stented coronary segment. All blocks were randomly labelled by an external observer with a numbered code, which was placed in a sealed envelope until the end of all data collection and analysis.

5.2.7 Slide preparation

Each resin block containing the stented artery segment was removed from the plastic tube using a scalpel. The distal end of the resin block was stuck to a glass

microscope slide using toughened acrylic adhesive (159-3991, RS). This produced superior bonding compared to Super Glue (Loctite®). Using a diamond edged rotary saw (Buehler Isomet® 1000), the slide was mounted on a chuck and a thin section cut 300-400 µm from the slide end. The new distal end of the resin block was then stuck to another slide with adhesive. This process was repeated to produce 6 good quality sections from the distal to proximal portion of the stent. Once cut, the sections were ground and polished by hand using a grinder/polisher (Buehler Metaserv® 2000) to reduce the thickness of the section to 10 µm and give a uniform surface for staining and microscopic evaluation. Sections were stained using haematoxylin-eosin and modified Carstairs' stain. Images were acquired using a Leica DM LB2 microscope and Leica DFC320 digital camera. Blinded histological analysis was performed using computerized morphometry software (Image-Pro Plus, Cybernetics).

5.2.8 Histomorphometric analysis

Histomorphometric analysis was according to Schwartz *et al*. (274) (Figure 5.5). Neointimal thickness was calculated as the mean distance from each stent strut to lumen; neointimal area was calculated as stent area minus lumen area; diameter stenosis was calculated as 100 × (1 − lumen area / IEL area). Binary ISR was defined as ≥ 50% diameter stenosis. Stent endothelialisation score was defined as the extent of the circumference of the arterial lumen covered by ECs and graded from 1 to 3 (1 = 25%; 2 = 25% − 75%; 3 ≥ 75%). Inflammation was graded as 0, none; 1, scattered inflammatory cells; 2, inflammatory cells encompassing 50% of a strut in at least 25% to 50% of the circumference of the artery; 3,

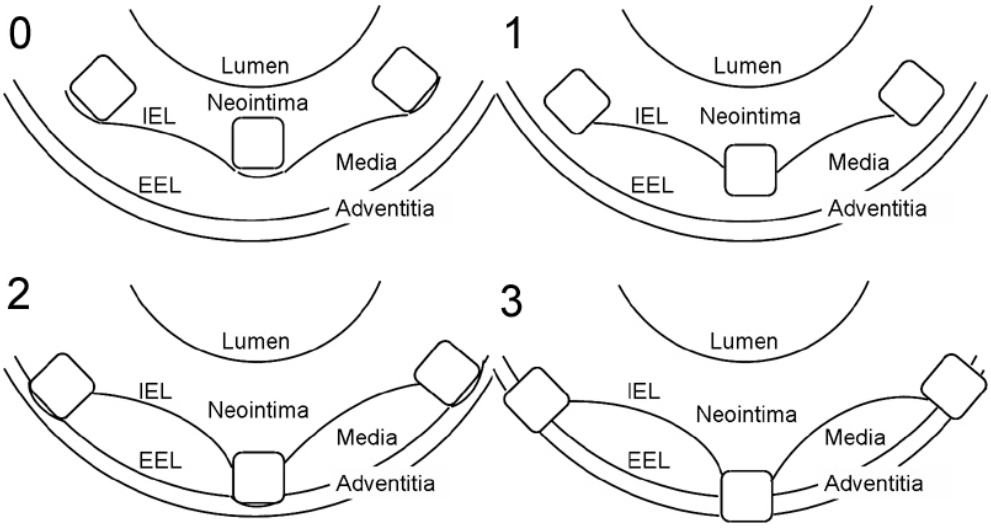

Figure 5.5 Histomorphometry and injury score. Injury score was defined as 0, IEL intact, media compressed; 1, IEL lacerated, media compressed; 2, Media lacerated, EEL intact; 3, EEL lacerated. Stent struts are represented by rounded squares.

inflammatory cells surrounding a strut in at least 25% to 50% of the circumference of the artery. The intimal fibrin content was graded as 0, no residual fibrin; 1, focal regions of residual fibrin involving any portion of the artery or moderate fibrin deposition adjacent to the strut involving < 25% of the circumference of the artery; 2, moderate fibrin involving > 25% of the circumference of the artery or heavy deposition involving < 25% of the circumference of the artery; 3, heavy fibrin deposition involving > 25% of the circumference of the artery. All sections were examined for presence of uncovered stent struts and evidence of in-stent thrombus was determined throughout the entire stent length.

5.2.9 WBA studies

As succinobucol was to be used for the first time as a novel DES coating, its effect on WBA was investigated, primarily for safety purposes. This part of the study was performed following the premature death of two pigs, to ensure that succinobucol was not exerting a prothrombotic effect. WBA was performed according to the method described in section 2.2.3. The effect of succinobucol (1-100 µg/ml) on WBA was determined using fresh porcine blood, withdrawn from the main pulmonary artery immediately after euthanasia for 4 pigs. 1 µl of 1, 10 and 100 mg/ml succinobucol (in 99.5% ethanol) was added to 1 ml of whole blood:0.9% saline (1:1), 1 minute prior to addition of agonist. 3 µg/ml collagen and 20 µM ADP were used as platelet agonists. A higher concentration of ADP was used in this study because pigs were taking clopidogrel, an ADP receptor antagonist. Vehicle (0.1% ethanol) had no effect on WBA.

5.2.10 Cell culture studies

Bovine pulmonary artery ECs, obtained from a local abbatoir, were seeded on to 6 well plates (up to passage 4) and grown to 70% confluence. Succinobucol or probucol was added to each well at concentrations (1-20 µmol/L) that have previously been shown to inhibit SMC proliferation (149;165;166). An additional well received the maximum amount of vehicle (0.3% dimethyl sulphoxide). After 24 hours, the well plates were inspected and photographed, taking account of any evidence of cytotoxicity. The medium was removed, and adherent cells were dislodged using trypLE Express (Invitrogen, UK). Trypan blue (0.07%) was added to the cell suspension and cells counted using a hemocytometer. The percentage of the counted cells not stained by trypan blue is reported as the

percent viable cells. Identical experiments were performed using abbatoir derived bovine pulmonary artery SMCs, grown to 90% confluence. In additional experiments, 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor) 0.2 µM was used to generate oxidative stress and administered to bovine aortic SMCs alone and in combination with succinobucol or probucol. Originally, the effect of succinobucol on cell proliferation was to be studied, however the proinflammatory actions of the SES found *in vivo* and the damaging effects noted in cell cultures led to a change in the protocol to address cytotoxicity and viability.

5.2.11 Toxicology studies

Given that succinobucol is a close analogue of probucol, which is known to prolong the QT interval, the effects of succinobucol on the surface electrocardiogram was assessed. 500 µg succinobucol (an approximation of the mass of drug carried on a SES) was infused into a coronary artery of a pig, not included in any other study. Electrocardiographic and haemodynamic monitoring was performed throughout, looking specifically for evidence of ischaemia, dysrhythmias or QT interval prolongation. Succinobucol concentration in the blood (withdrawn from the pulmonary artery) was measured 1 hour and 1 day after implantation of 2 SES, using HPLC.

5.2.12 Statistical analysis

Histomorphometric data for each stent was the mean of six sections from proximal to distal end. The endothelial score, inflammatory score and fibrin score were the mean of two sections per stent. Group means were calculated and

then compared by one-way analysis of variance and post-hoc Dunnett's test. For WBA experiments, aggregation in response to ADP was severely reduced by clopidogrel, therefore values are absolute impedance. For the efficacy study, a sample size of 9 per group was calculated to provide 80% power to detect a treatment difference of 30% between groups at a two-sided 0.05 significance level, based on the assumption that the SD of the response variable (neointimal area) was 20% as shown by similar studies in the porcine model (58;272). All data are expressed as mean ± SEM unless otherwise stated. Significance was established by a value of $p < 0.05$. Statistical analysis was performed using the SPSS statistical software package 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

5.3 Results

5.3.1 Optimisation of SES coating

0.5% (5 mg/ml), 1% (10 mg/ml) and 2% (20 mg/ml) succinobucol solutions in 99.5% ethanol were sprayed onto a Yukon® stent and examined using scanning electron microscopy. 1% succinobucol coating solution produced a smooth uniform complete drug layer, optimal for the Yukon® DES delivery system and was therefore considered most appropriate for preclinical assessment (Figure 5.6). 0.5% succinobucol produced incomplete strut coverage, with some areas of stent sparsely covered with drug. 2% succinobucol produced excessive coverage, with surplus drug deposition between struts and crystallisation of drug (10-25 µm diameter) present on the surface of the stent, which may become hazardous after balloon dilatation if distal embolisation were to occur. The coating device provided drug deposition on the exterior (abluminal) surface of the stent struts, whilst the interior (adluminal) surface remained essentially uncoated with drug (Figure 5.7).

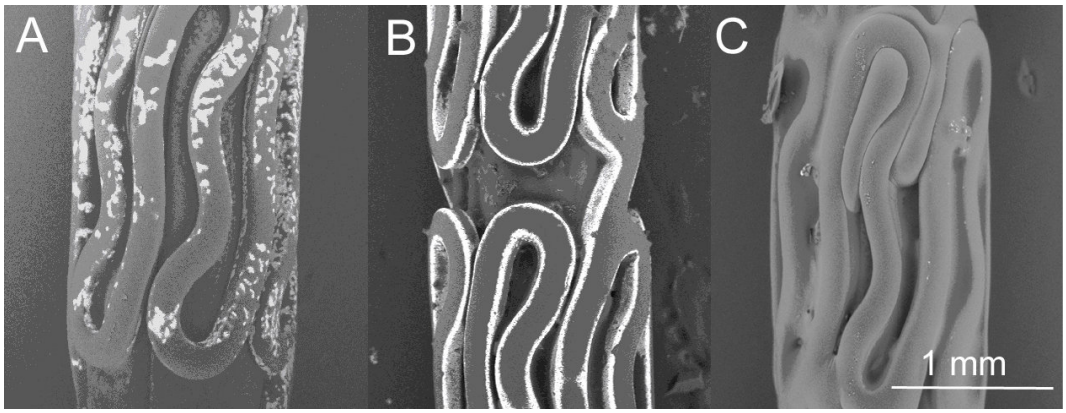

Figure 5.6 A comparison of stent coverage for succinobucol coating solutions. A, 0.5% succinobucol provided suboptimal coverage with drug (incomplete coverage areas are white). B, 1% succinobucol produced optimal strut coverage. C, 2% succinobucol caused surplus drug deposition and crystallisation (SEM, × 100 magnification).

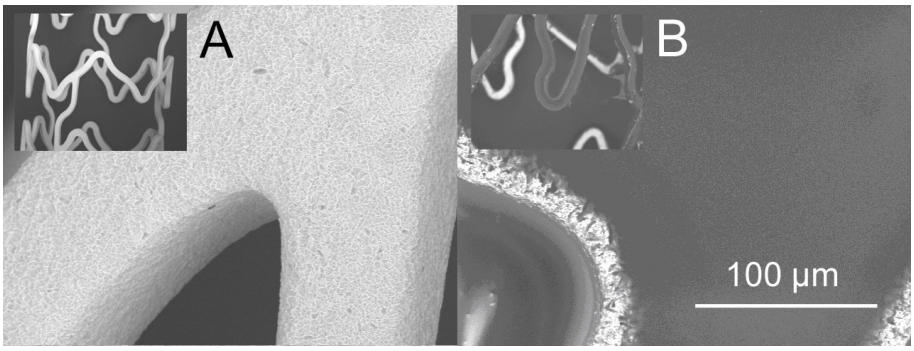

Figure 5.7 A comparison of uncoated and 1% succinobucol coated stents. A, BMS with microporous surface designed to act as a reservoir for drug deposition (insert: expanded BMS). B, 1% SES with smooth uniform drug layer (insert: SES with drug deposition on abluminal surface). Main figures (SEM, × 1000 magnification); inserts (SEM, × 100 magnification).

5.3.2 Drug loading of SES

The total drug loaded on the SES using 1% succinobucol was measured in 4 stents. The coating process produced a mass of 465 ± 61 µg succinobucol per SES. The drug loading of the RES using 2% rapamycin has been reported as producing 479 ± 26 µg rapamycin/cm^2 (265), which is equivalent to 842.7 ± 46 µg for a 3.5 mm × 16 mm stent.

5.3.3 *In vivo* study plan

6 pigs underwent coronary stenting to investigate *in vivo* pharmacokinetics of the SES and 19 pigs underwent coronary stenting as part of the *in vivo* efficacy study. The study plan is shown in Table 5.1. No pigs died prematurely during the pharmacokinetic study. There were 2 unexpected premature deaths during the efficacy study. One pig died the first night after successful implantation of a RES and SRES. Post-mortem examination identified no gross evidence of myocardial infarction, however careful dissection of the coronary arteries revealed occlusive stent thrombosis (Figure 5.8) in both stents. A second pig died on the operating table during recovery from general anaesthesia. Electrocardiographic monitoring displayed ventricular fibrillation during cardiac arrest (Figure 5.9). Post-mortem examination showed no gross cardiac abnormality, but there was evidence of occlusive stent thrombosis in both stents (SES and SRES). The diagnosis for both premature deaths was acute stent thrombosis (within 24 hours). 17 pigs completed the 28 day efficacy study and 41 stents were available for histological evaluation.

Table 5.1 *In vivo* study plan. Premature deaths are highlighted in grey (pig 2 and 5 in efficacy study). Each pig received 2-3 stents.

Pig	LAD	LCx	RCA
Pharmacokinetic study			
1 (1 hour)	SES	SES	SES
2 (1 day)		SES	SES
3 (3 days)	SES		SES
4 (7 days)	SES	SES	
5 (14 days)	SES		SES
6 (28 days)	SES	SES	
Efficacy study			
1	SES	BMS	
2	SRES	RES	
3	BMS		RES
4	RES		BMS
5	SES		SRES
6		RES	BMS
7	BMS	RES	
8	BMS		RES
9	BMS	RES	
10	RES	SES	
11	RES	SES	
12	SES	SRES	
13	SES	BMS	RES
14	BMS	SES	RES
15	SRES	SRES	BMS
16	SRES	SES	SRES
17	SES	SRES	SRES
18	SRES	SRES	SES
19	SRES	BMS	SES

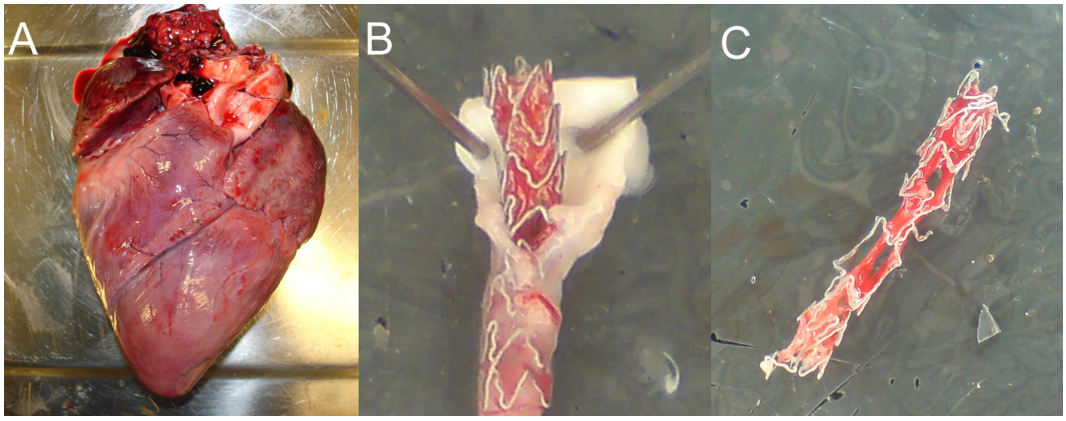

Figure 5.8 Fatal stent thrombosis in the pig. A, Gross cardiac specimen showing no evidence of myocardial infarction. B and C, Dissection of coronary artery revealed occlusive stent thrombosis.

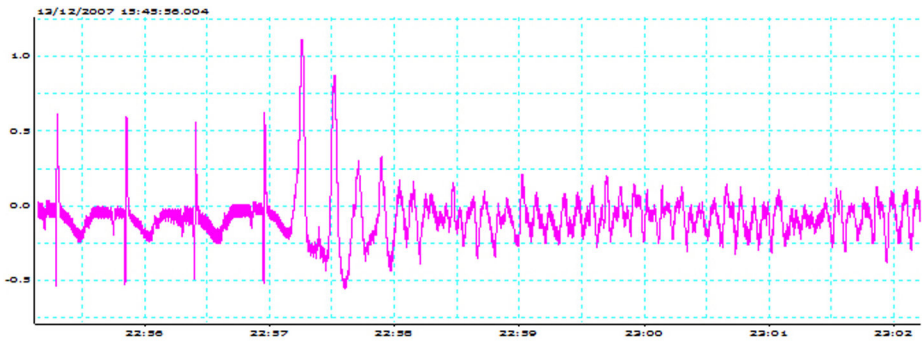

Figure 5.9 Ventricular fibrillation during cardiac arrest after stent implantation. The electrocardiograph shows 4 normal sinus beats followed by an R on T phenomenon inducing fatal ventricular fibrillation.

5.3.4 *In vivo* release profile of SES

To determine *in vivo* release characteristics of succinobucol from the novel SES, 13 SES were deployed in 6 pigs, using the techniques as previously described. Each pig received 2-3 stents in different coronary arteries (n = 2-3 for each time point) and specimens were harvested 1 hour, 1, 3, 7, 14 and 28 days after stent implantation. The SES provided sustained *in vivo* drug release for up to 4 weeks (Figure 5.10). 59.4% of the total succinobucol loaded on the SES was eluted during the first week and 81.0% was eluted after 28 days. This was very similar to the release profile of the RES, which retards drug release for greater than 3 weeks, with around two-thirds released during the first week (272;273). Succinobucol concentration in coronary artery tissue immediately surrounding the stent was quantified at 1 hour, 1, 3, 7, 14 and 28 days after stent implantation (Figure 5.11). At 1 hour, succinobucol concentration in surrounding artery tissue was 22.4 ng/mg. At 1 day, succinobucol tissue concentration peaked at 825.9 ng/mg. Succinobucol concentration in surrounding artery tissue was > 200 ng/mg for the remainder of the study. At 28 days, succinobucol tissue concentration was 242.2 ng/mg.

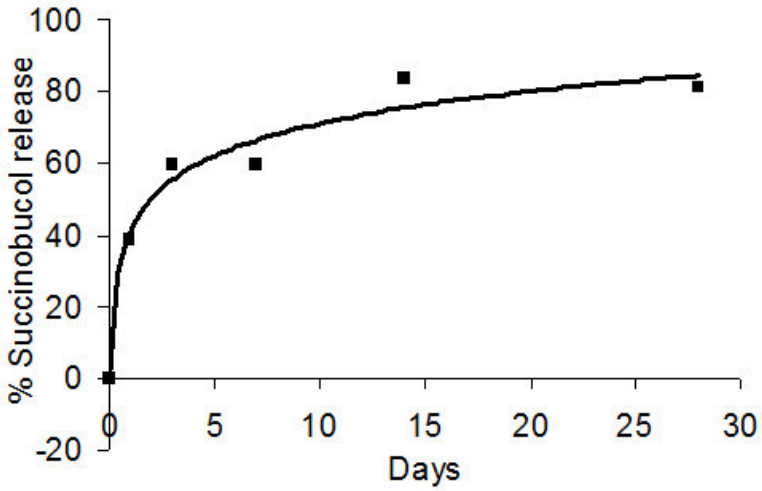

Figure 5.10 *In vivo* release profile of SES. Succinobucol elution was controlled over 4 weeks, with the majority of drug released in the first week. Data points represent % succinobucol released as a percentage of total drug loaded on the SES.

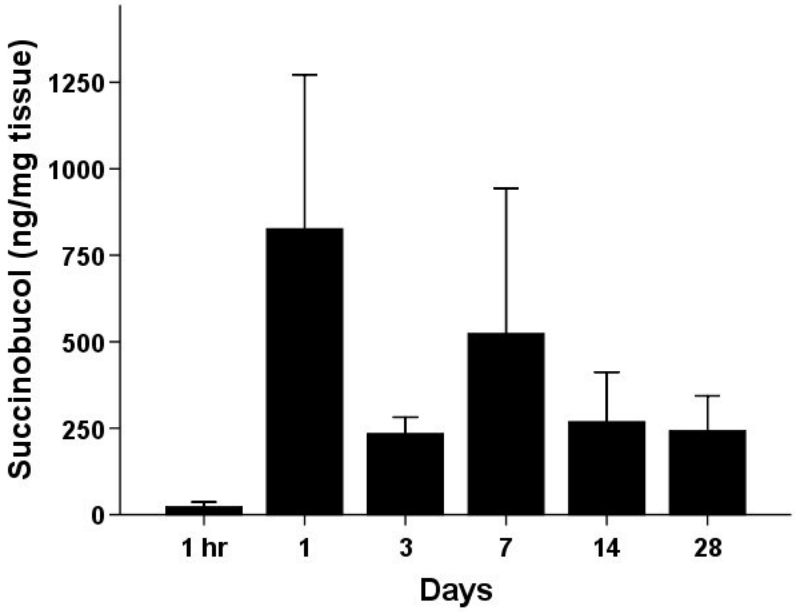

Figure 5.11 Succinobucol tissue concentration after SES implantation. Succinobucol concentration in surrounding artery tissue peaked 1 day after implantation and was maintained for the duration of the study period (n = 2).

5.3.5 Efficacy results

In total, 41 stents were implanted in the coronary arteries of 17 pigs for evaluation of efficacy at 28 days (BMS, n = 11; SES, n =10; RES, n = 10; SRES, n = 10). Representative photomicrographs from each group are shown in Figure 5.12.

Injury score

There was no significant difference in injury score between groups (Figure 5.13).

Vessel size

EEL area was increased in the RES group, compared with all other groups (7.84 ± 0.30 mm^2, 7.52 ± 0.28 mm^2, 9.03 ± 0.30 mm^2 and 7.61 ± 0.22 mm^2 for BMS, SES, RES and SRES, respectively, $p < 0.05$). IEL area (6.74 ± 0.27 mm^2, 6.35 ± 0.30 mm^2, 7.94 ± 0.29 mm^2 and 6.56 ± 0.24 mm^2) and stent area (6.35 ± 0.24 mm^2, 6.01 ± 0.27 mm^2, 7.54 ± 0.29 mm^2 and 6.29 ± 0.24 mm^2) were also increased in the RES group (BMS, SES, RES and SRES, respectively, both $p < 0.05$). Other group comparisons were not statistically significant.

Medial area

There was no difference in medial area at 28 days between groups (1.10 ± 0.06 mm^2, 1.17 ± 0.07 mm^2, 1.10 ± 0.06 mm^2 and 1.05 ± 0.06 mm^2 for BMS, SES, RES and RSES, respectively, p = NS).

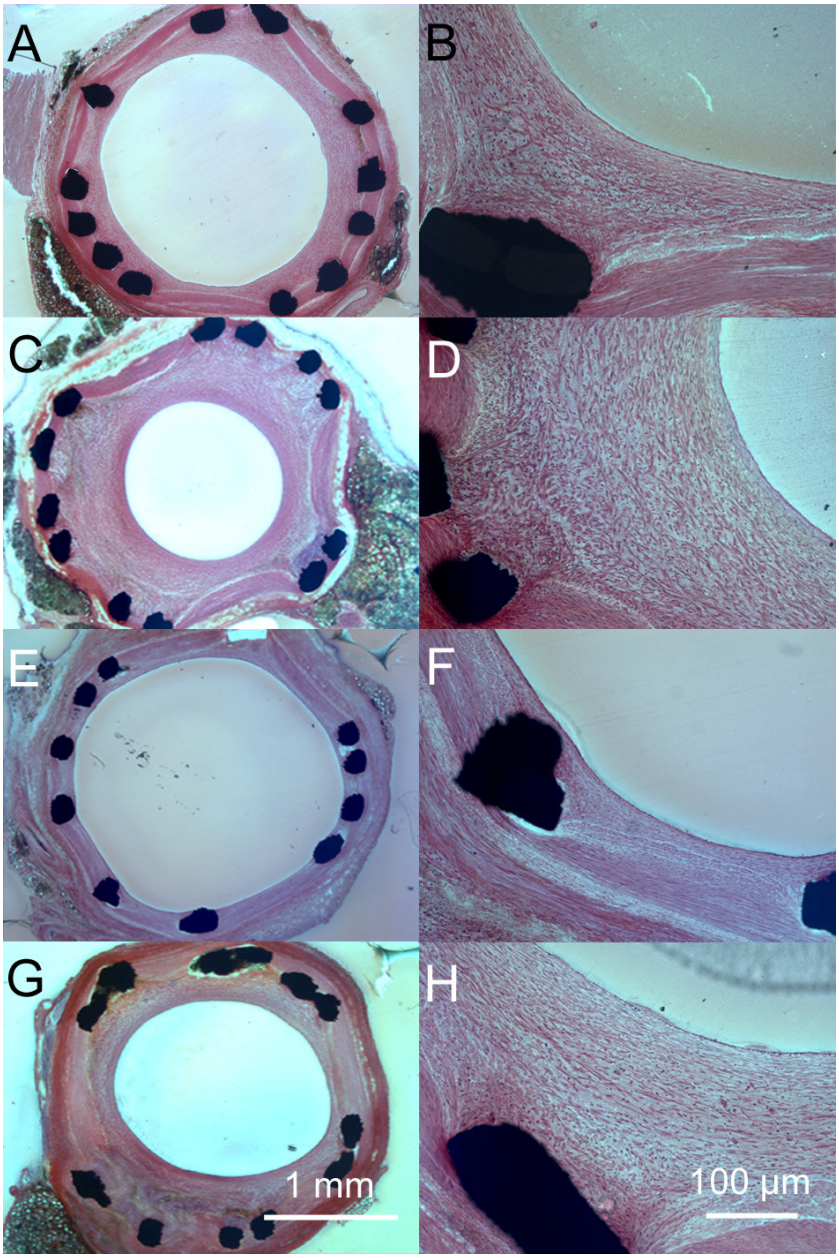

Figure 5.12 Representative photomicrographs of stent sections. BMS (A,B) had moderate neointimal formation; SES (C,D) had the most extensive neointima; RES (E,F) had minimal neointimal growth; SRES (G,H) had moderate neointima similar to BMS. A, C, E, G (× 2 magnification); B, D, F, G (× 10 magnification).

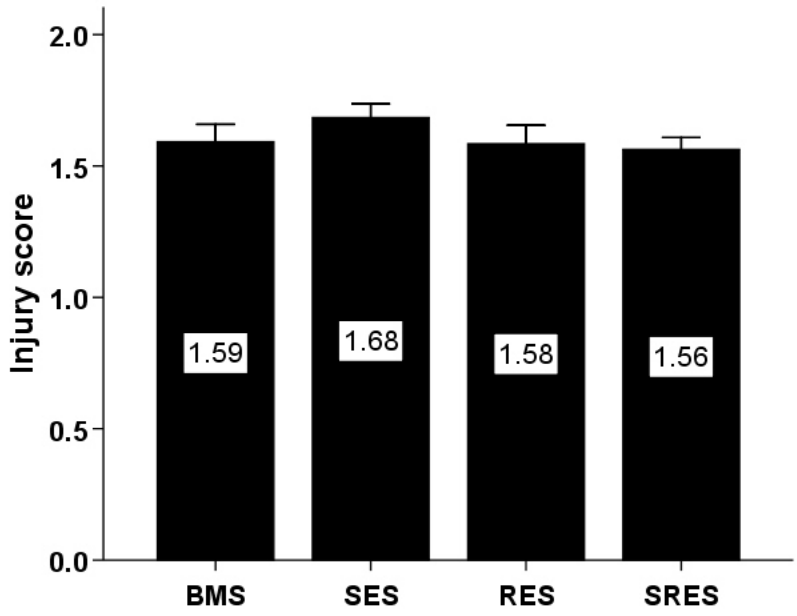

Figure 5.13 A comparison of injury score between groups. No significant differences were present (n = 10-11), p = NS.

Neointimal area and thickness

Neointimal area was increased significantly in the SES group, whereas there was a trend to decreased neointimal area in the RES group (Figure 5.14). Similar results were evident for neointimal thickness (Figure 5.15). There were no significant differences in neointimal area/medial area. Neointimal area/IEL area and neointimal area/EEL area were both increased significantly in the SES, compared with BMS (both $p < 0.01$). In all groups, the neointima was composed almost entirely of SMCs with inflammatory cells around the struts and little extracellular matrix (Figure 5.16).

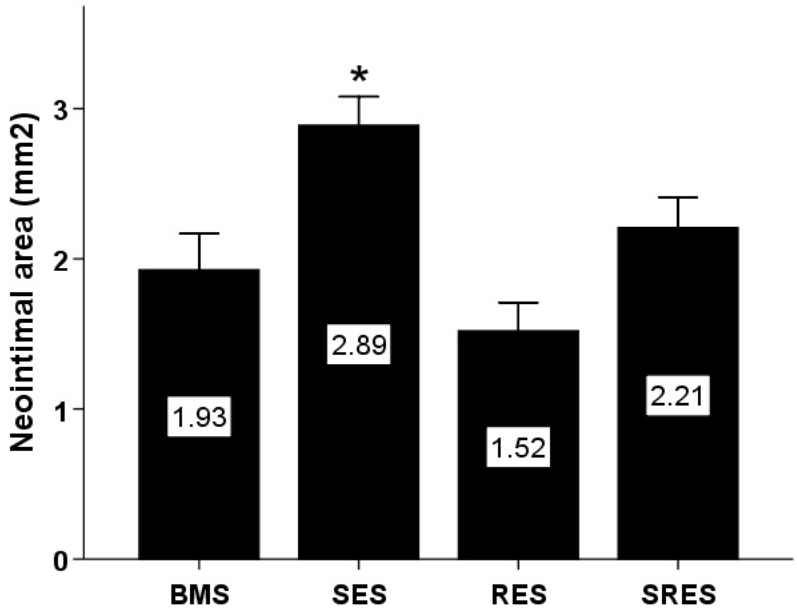

Figure 5.14 A comparison of neointimal area between groups. Neointimal area was increased significantly in SES, compared with BMS (n = 10-11), * p < 0.01 vs. BMS.

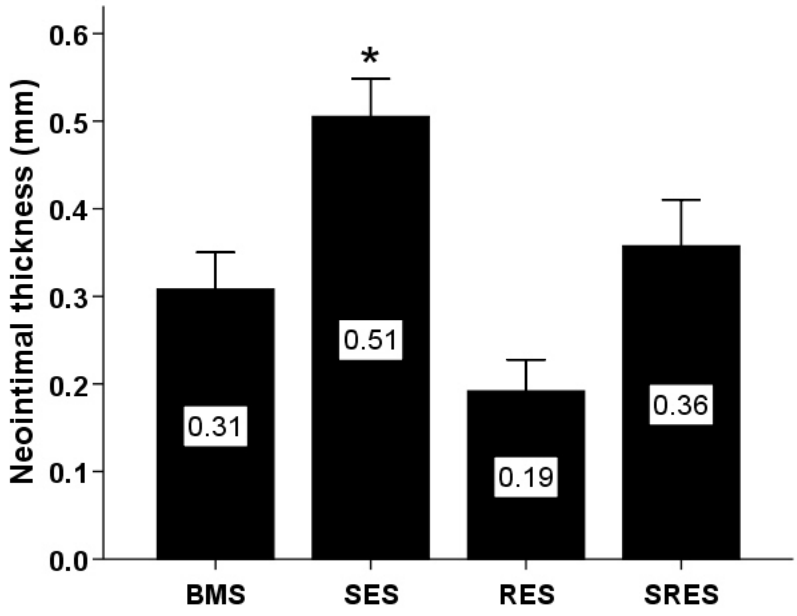

Figure 5.15 A comparison of neointimal thickness between groups. Neointimal thickness was increased significantly in SES, compared with BMS (n = 10-11), * p < 0.01 vs. BMS.

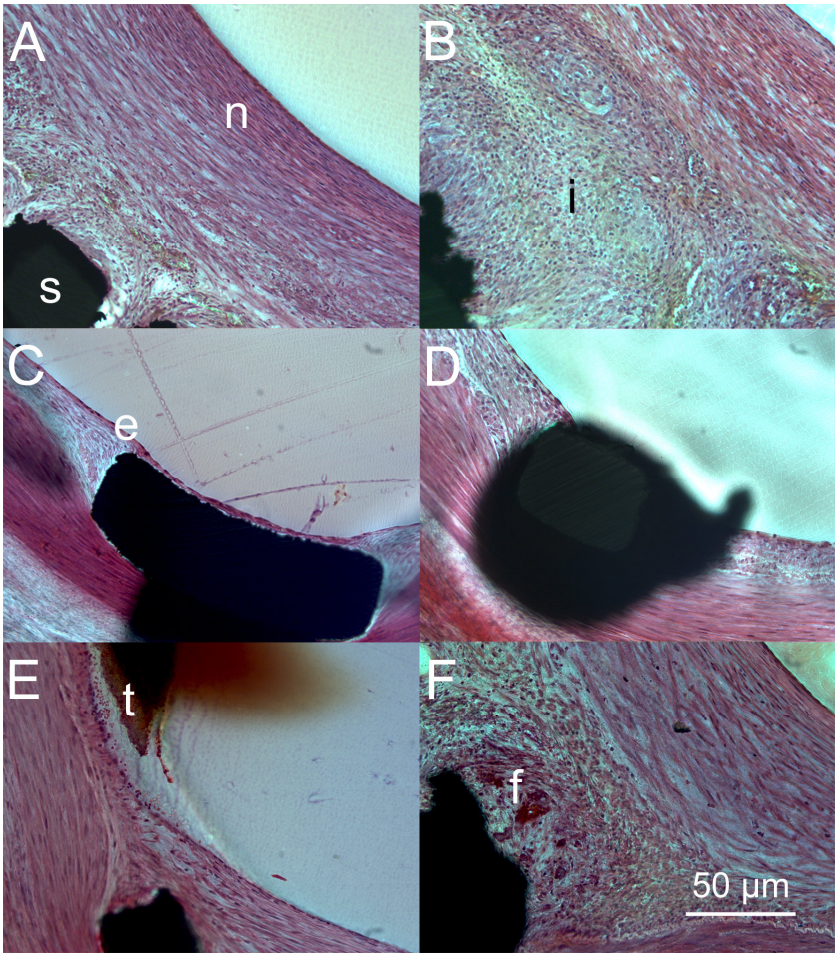

Figure 5.16 Representative photomicrographs of stent sections (high power). A, Neointima (n) over stent struts (s) was made up predominantly of SMCs. B, Inflammatory cells (i) around a strut in SES. C, Thin endothelial monolayer (e) over the adluminal surface of a stent strut in RES. D, Uncovered strut with interruption of endothelium. E, Luminal thrombus (t) over an area of poor re-endothelialization. F, Fibrin (f) deposition around a strut in SES. All sections (× 20 magnification).

Diameter stenosis

SES caused a significant increase in diameter stenosis, whereas the decreased diameter stenosis in the RES group was not significant ($p = 0.17$) (Figure 5.17). The rate of binary ISR (> 50% diameter stenosis) measured by histological analysis was 9.1%, 60%, 0% and 20% for BMS, SES, RES and SRES groups, respectively ($p < 0.01$).

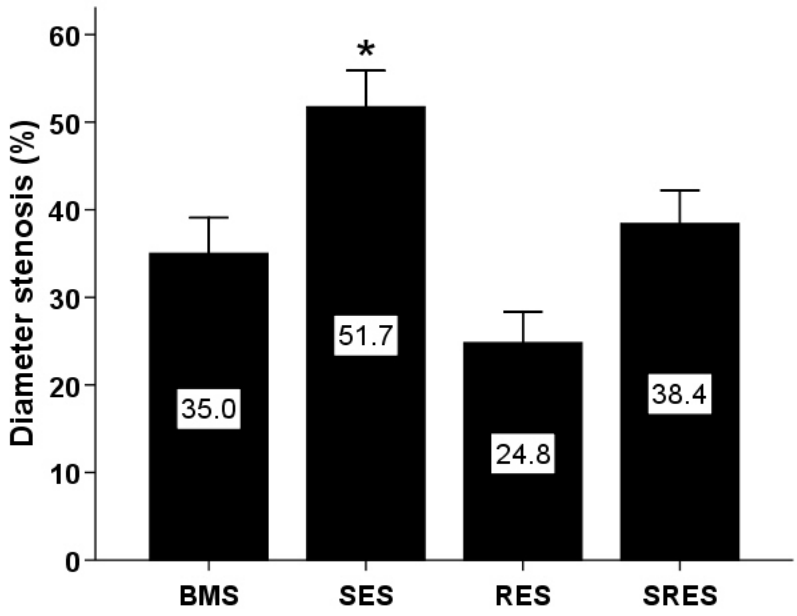

Figure 5.17 A comparison of diameter stenosis between groups. Diameter stenosis was increased significantly in SES, compared with BMS (n = 10-11), * $p < 0.05$ vs. BMS.

Lumen area

The lumen area was increased significantly in the RES group and the decrease in lumen area in the SES group was of borderline significance (Figure 5.18).

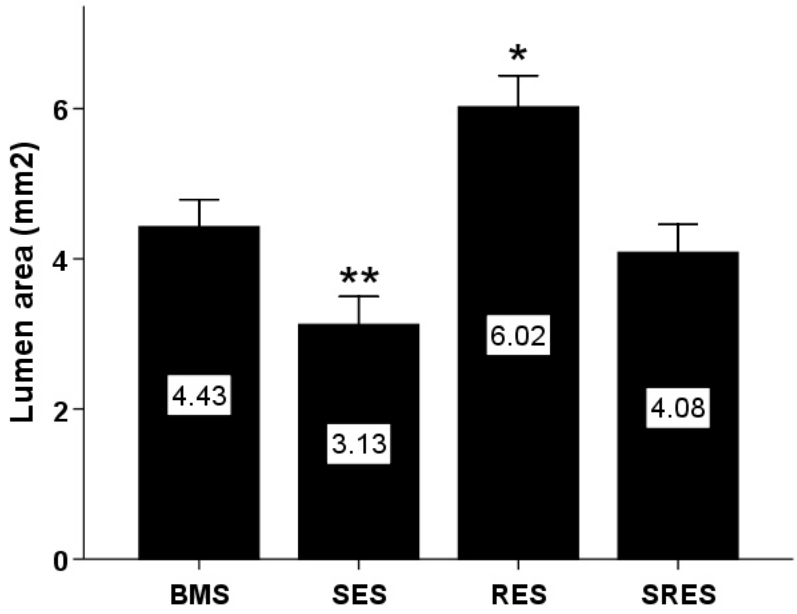

Figure 5.18 A comparison of lumen area between groups. Lumen area was increased significantly in RES and decreased of borderline significance in SES, compared with BMS (n = 10-11), * $p < 0.05$, ** $p = 0.05$ vs. BMS.

Endothelial regeneration

28 days after implantation, all BMS and SES had an endothelial score of 3. Two RES and two SRES had an endothelial score of 2.5 (derived from mean of two sections per stent). This resulted in a small but non-significant reduction in endothelial score in both rapamycin groups (p = 0.30) (Table 5.2). The mean semiquantitative percentage of circumference covered by endothelium was 98.3 ± 1.0%, 98.3 ± 1.1%, 96.4 ± 1.8% and 96.0 ± 2.3% in BMS, SES, RES and SRES, respectively, p = NS. The rate of stents with any uncovered stent struts was 9.1%, 0%, 30% and 0% for BMS, SES, RES and SRES groups, respectively (p = 0.08).

In-stent thrombus and fibrin deposition

The rate of stents with in-stent thrombus was 27.3%, 10%, 20% and 20% for BMS, SES, RES and SRES groups, respectively (p = 0.81). Fibrin scores were markedly higher in both succinobucol groups (Table 5.2).

Inflammation

Inflammatory infiltrates present in the sections after 28 days were significantly increased in SES and significantly reduced in RES (Table 5.2). Inflammatory cells were most prevalent close to the stent struts (Figure 5.16).

Table 5.2. Healing and inflammation.

Group	Endothelial Score	Fibrin Score	Inflammatory Score
BMS (n=11)	3.00 ± 0.00	0.09 ± 0.20	1.96 ± 0.42
SES (n=10)	3.00 ± 0.00	0.95 ± 0.69*	2.35 ± 0.41**
RES (n=10)	2.90 ± 0.21	0.15 ± 0.24	1.55 ± 0.28**
SRES (n=10)	2.90 ± 0.21	1.25 ± 0.83*	2.00 ± 0.00

* $p < 0.005$ vs. BMS; ** $p < 0.05$ vs. BMS.

5.3.6 *In vivo* toxicology of succinobucol

500 µg succinobucol infused directly into the left anterior descending coronary artery over 10 minutes (50 µg/ml at 1 ml/min) was well tolerated with no evidence of ischaemia, thrombosis or QT prolongation. The infusion was repeated after BMS implantation (to induce endothelial injury) with no adverse effects. Vehicle (0.5% alcohol) was tested without incident. The SES caused no significant change in QT_c interval (410.3 ± 14.5 ms before vs. 422.3 ± 7.5 ms after SES implantation, p = NS). No succinobucol was detected in blood samples obtained from the pulmonary artery 1 h or 1 day after deployment of 2 SES.

5.3.7 Effect of succinobucol on WBA

There was no significant effect of succinobucol (1-100 µg/ml) on WBA compared with control, in response to 3 µg/ml collagen (Figure 5.19) or 20 µM ADP in porcine blood (Figure 5.20). All pigs were treated with aspirin and clopidogrel.

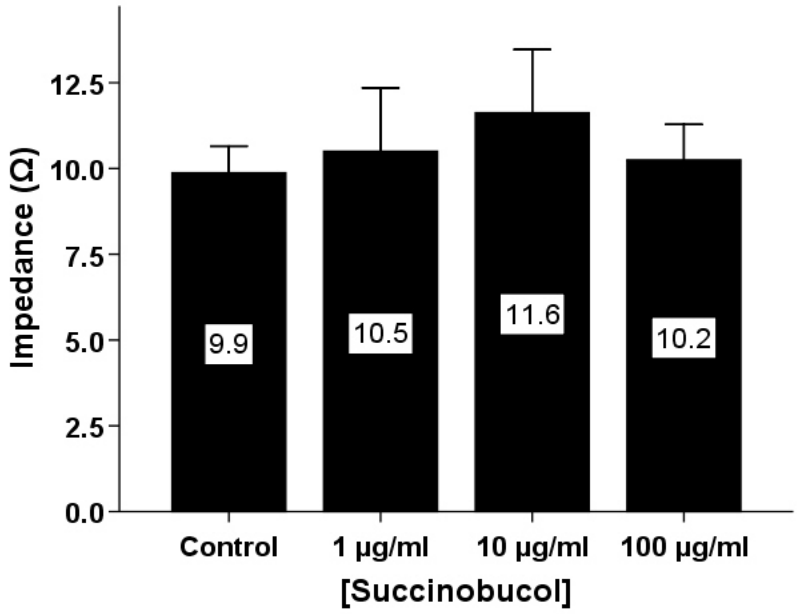

Figure 5.19 The effect of succinobucol on WBA in response to collagen. Succinobucol (1-100 µM) had no effect on WBA in response to 3 µg/ml collagen in porcine blood (n = 4), p = NS.

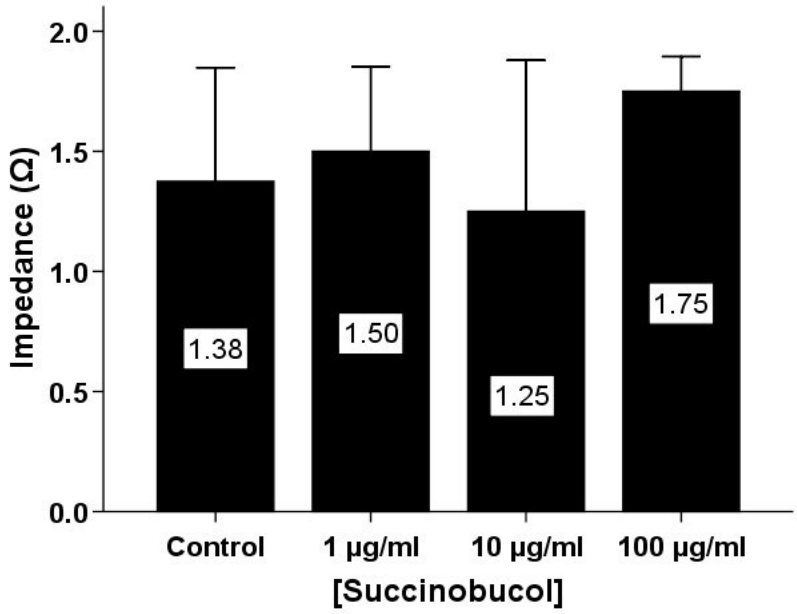

Figure 5.20 The effect of succinobucol on WBA in response to ADP. Succinobucol (1-100 µM) had no effect on WBA in response to 20 µM ADP in porcine blood (n = 4), p = NS.

5.3.8 Effect of succinobucol on cultured ECs and SMCs

Succinobucol caused marked toxicity to cultured bovine pulmonary artery ECs and SMCs.

EC culture

After 24 hours, 1 µM succinobucol caused a few ECs to detach from the well plates in culture, whereas above 5 µM, succinobucol caused almost all ECs to lift off the plate (Figures 5.21). Trypan blue staining showed that adherent ECs remained viable up to 5 µM succinobucol. However, with higher concentrations of succinobucol, there was a significant decline in EC viability (Figure 5.22).

SMC culture

After 24 hours, 1 µM succinobucol caused a few cells to detach from the well plates in culture, whereas above 10 µM, succinobucol caused almost all SMCs to lift off the plate (Figures 5.23). The adherent SMCs had significantly reduced viability in 1, 5 and 20 µM succinobucol (Figure 5.24). Probucol was considerably less toxic to SMCs than succinobucol, with minor detachment of SMCs occurring only in 20 µM probucol (Figure 5.25). Probucol had no significant deleterious effect on SMC viability (Figure 5.26).

Protection against SIN-1

0.2 µM SIN-1 reduced viability of ECs and SMCs. 1 µM Succinobucol did not protect against SIN-1-induced toxicity, whereas 1 µM probucol provided some protection (Figure 5.27).

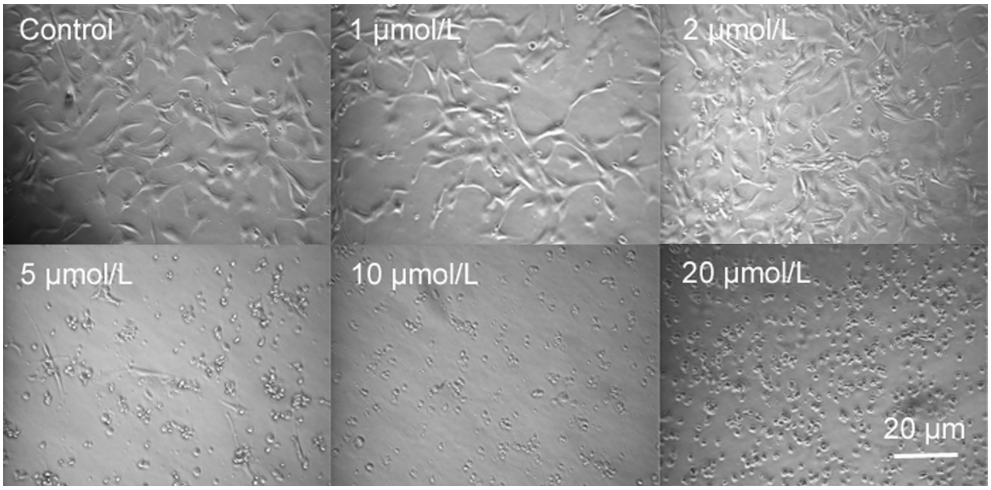

Figure 5.21 The effect of succinobucol on cultured ECs. After 24 hours, there was evidence of concentration-dependent cytotoxicity, resulting in total cell detachment in 10 µM and higher concentrations of succinobucol (magnification ×100).

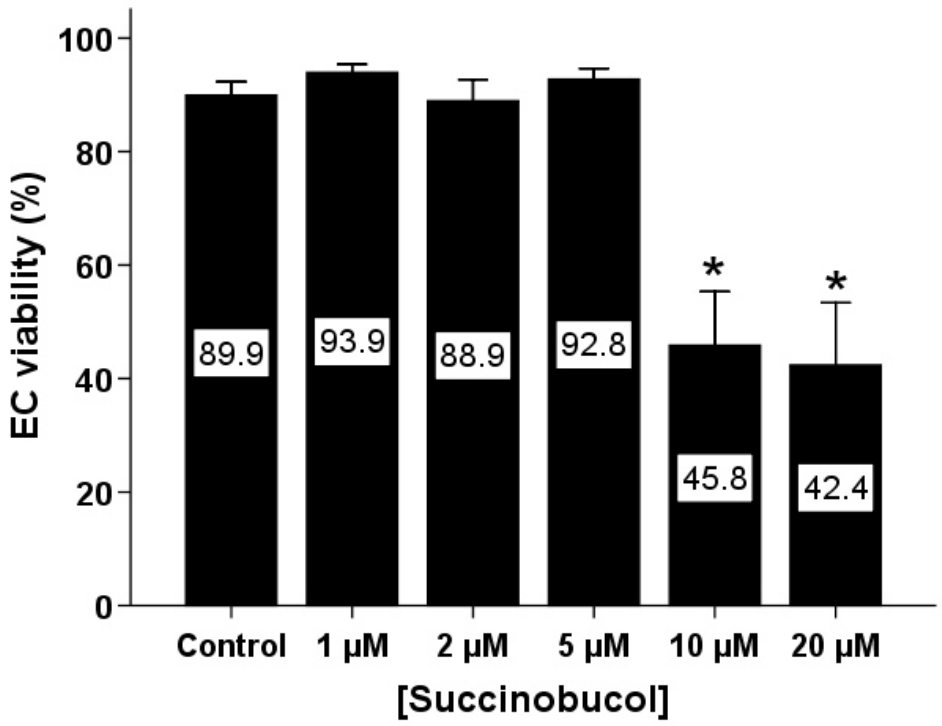

Figure 5.22 The effect of succinobucol on the viability of adherent ECs. Cell viability was reduced at concentrations of 10 µM and higher (n = 4-6), * $p < 0.05$ vs. control.

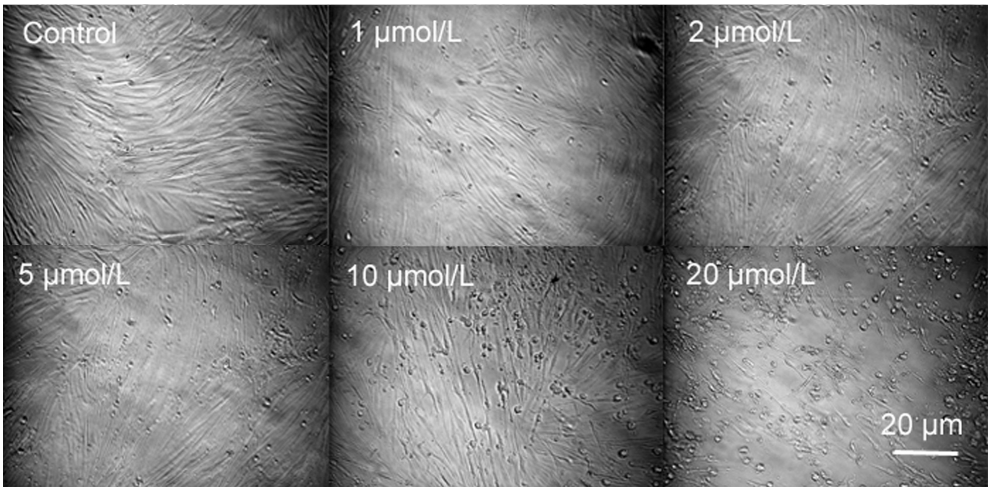

Figure 5.23 The effect of succinobucol on cultured SMCs. After 24 hours, there was evidence of concentration-dependent cytotoxicity, resulting in total cell detachment in 20 µM succinobucol (magnification ×100).

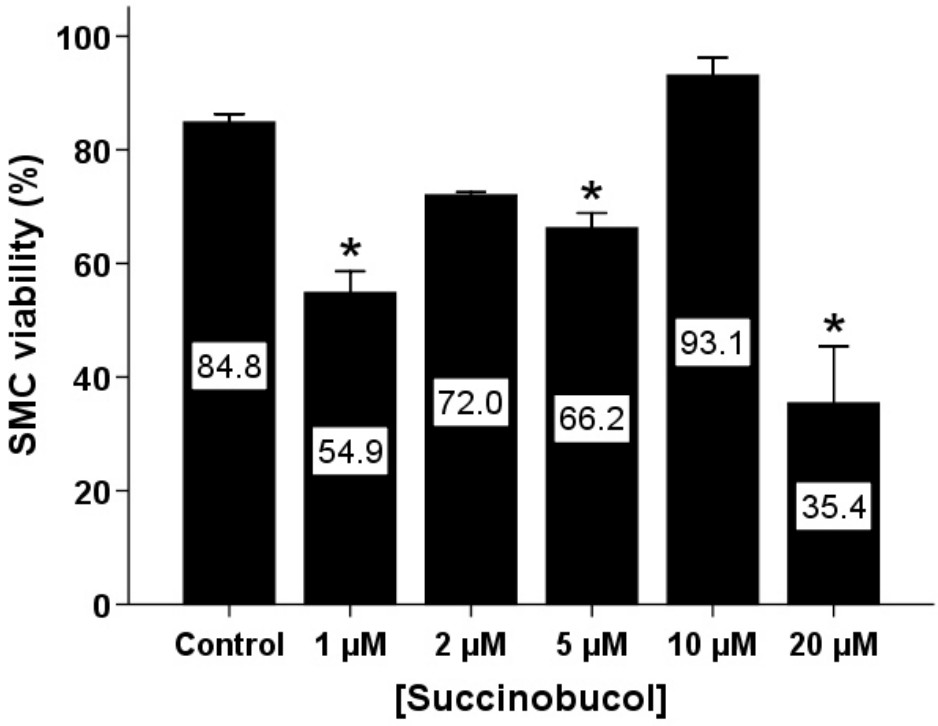

Figure 5.24 The effect of succinobucol on the viability of adherent SMCs. Cell viability was reduced in 1 µM, 5 µM and 20 µM (n =4-6), * $p < 0.05$ vs. control.

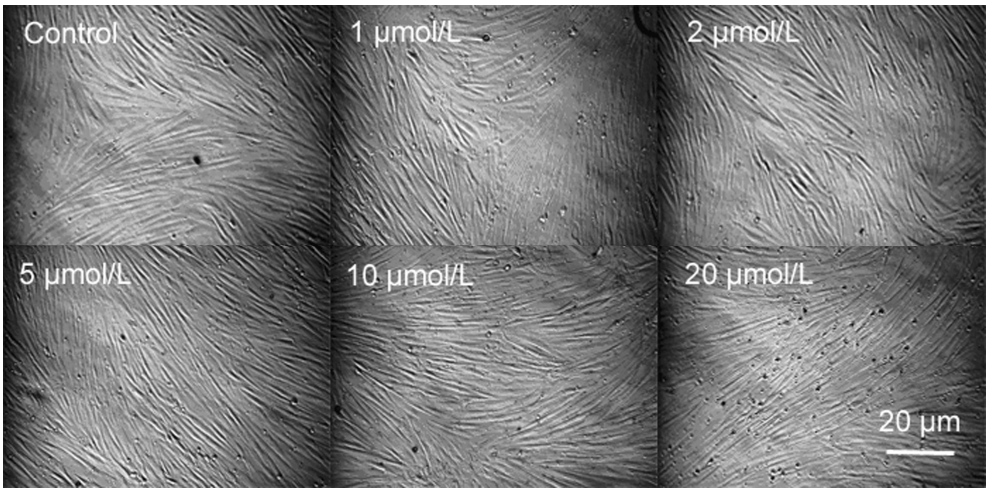

Figure 5.25 The effect of probucol on cultured SMCs. After 24 hours, there was maintained cell adherence with only minor cell detachment at the 20 µM probucol (magnification ×100).

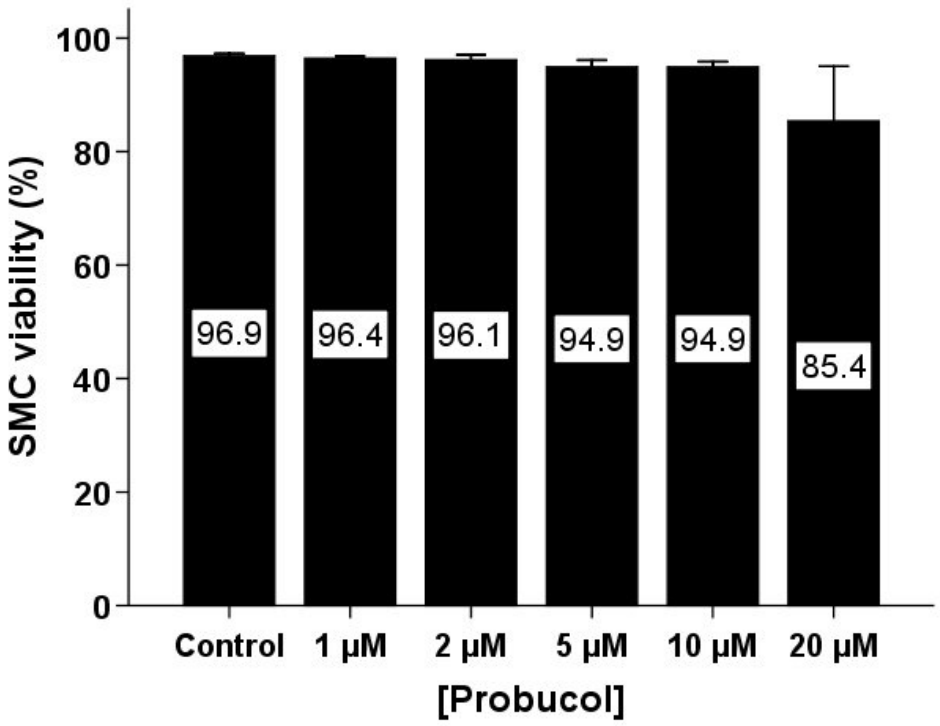

Figure 5.26 The effect of probucol on the viability of adherent SMCs. Probucol had no significant effect on the viability of remaining adherent SMCs (n = 4-5), p = NS.

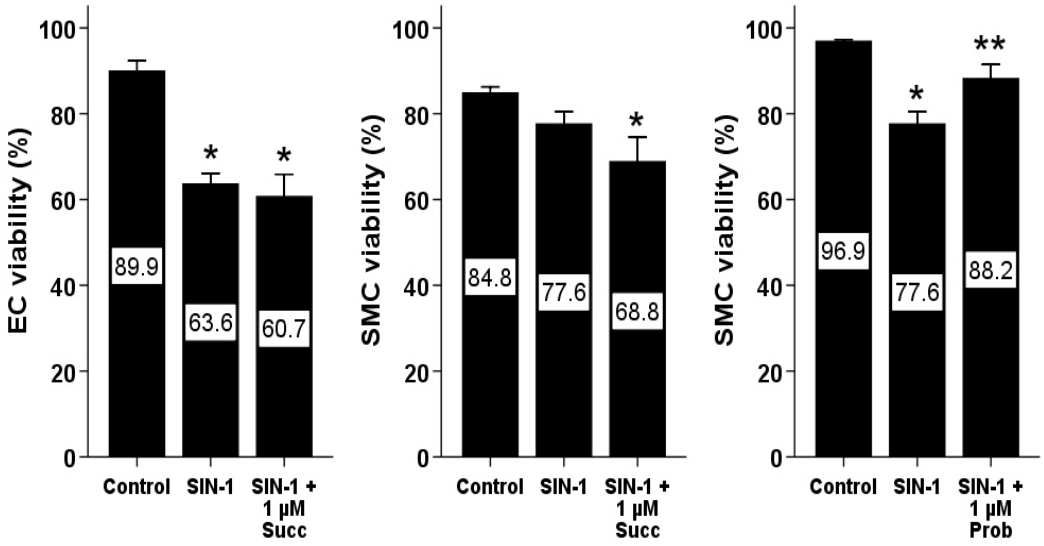

Figure 5.27 The effect of succinobucol and probucol on SIN-1-induced toxicity. Succinobucol was unable to protect ECs and SMCs from SIN-1-induced toxicity, however probucol provided some protection (n = 4-5), * $p < 0.01$ vs. control, ** $p < 0.05$ SIN-1 vs. SIN-1 + 1 µM probucol, p = NS all other comparisons.

5.4 Discussion

Since both oxidative stress (117;275;276) and inflammation (18;277) are responsible for increased neointimal formation, I chose to investigate the novel antioxidant and anti-inflammatory drug, succinobucol, coated on a stent, without a polymer to reduce the potential for adverse effects. Succinobucol reportedly had shown many properties which were extremely attractive as a potential DES coating. These included potent antiproliferative effects on SMCs (165;166), inhibitory effects on vascular inflammation (163-166), antiplatelet effects (268) and rapid uptake into cells (163), all of which were superior to probucol, the parent molecule with promising results for the prevention of ISR and promotion of endothelial healing, especially in animal models (149;150).

5.4.1 Effects of succinobucol

Drug coatings were sprayed onto unexpanded stents on a balloon, therefore drug deposition occurred onto the abluminal surface, which is ideal for a modern DES. Preferential abluminal DES coating allows more targeted drug release into the artery wall, reduces systemic exposure and limits potentially unforeseen drug toxicity to the ECs which repopulate the adluminal strut surface. This technique is now specifically employed in several modern DES currently in clinical use (278-280). Stent-based delivery of succinobucol was optimally controlled *in vivo*, despite the absence of a polymer, with around two-thirds of the drug released during the first week. This matches closely the period after stent deployment when ROS are released in large quantities from the artery wall (243).

However, surprisingly, local delivery of succinobucol increased inflammation and neointimal growth. Rate of ISR was also significantly increased in the SES group. Subsequently, succinobucol was found to destabilise cells in culture leading to cell detachment and loss of viability. However, cell toxicity *in vitro* was much less marked with probucol. Rapamycin is a potent inhibitor of SMC proliferation but has no deleterious effect on cell viability (281). I postulate that succinobucol exacerbated neointimal formation by causing direct cellular damage and inflammation, negating any putative local beneficial effects. Localised cellular deterioration and necrosis would have been likely to recruit inflammatory cells, as was observed. Degree of inflammation has been shown to correlate positively with neointimal growth (282) and this relationship has been reaffirmed by my results (Figure 5.28). Analysis of human peripheral artery

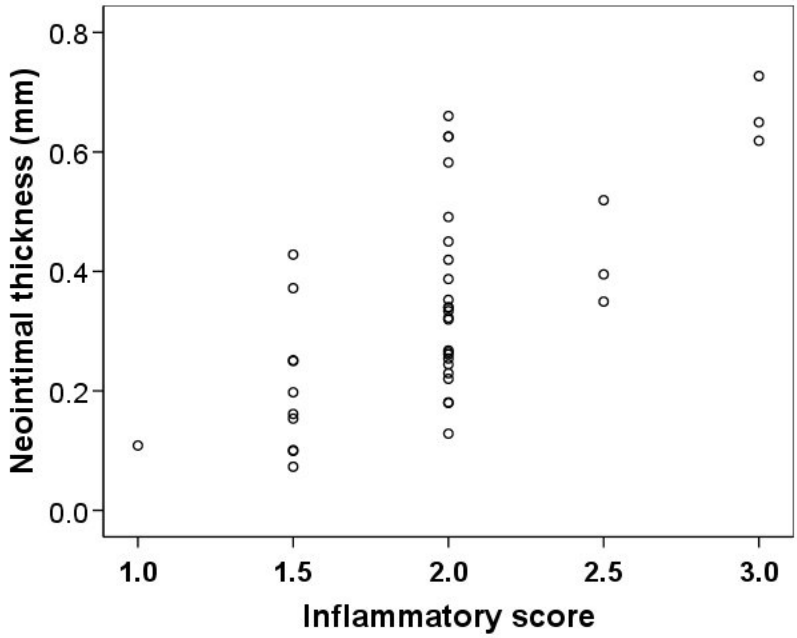

Figure 5.28 Scatter plot of inflammatory score and neointimal thickness. Neointimal thickness was strongly correlated with inflammatory score ($r = 0.67$, $p < 0.001$).

atherectomy specimens demonstrated that macrophages and leucocytes account for almost a quarter of neointimal cells in cases of ISR (283). Coronary atherectomy specimens from several patients who had received the paclitaxel derivative, 7-hexanoyltaxol (QP2)-eluting polymer stents, which also increased ISR, displayed widespread inflammatory aggregates and fibrin deposition in a SMC-rich neointima (284). These histological findings are analogous to those found with succinobucol in this study. Thus, I propose that the accumulation of inflammatory cells around SES struts caused increased production of chemotactic cytokines and growth factors, which led to the proliferation of SMCs and increased neointimal formation.

Succinobucol did not cause improved arterial healing, because increased fibrin and excessive inflammation around SES struts *in vivo* after 28 days are both

features of poor healing (274). Fibrin and platelet-rich thrombus adhere to metallic stent struts within hours of implantation. Normally, this progressively resolves over the following weeks, principally due to resorption by macrophages, which secrete a number of fibrinolytic enzymes (285;286). Near-total fibrin and thrombus resorption is a feature of complete arterial healing in both animal and human models. Increased fibrin deposition seen in both SES groups after 28 days in this study closely matched the late histological findings of other potent antirestenosis therapies that impede arterial healing, such as intracoronary radiation (287) and polymeric DES (62). Endothelial regeneration was difficult to assess in this model because, at the end of the study, the BMS were completely re-endothelialised. However, the toxic effects of succinobucol on cultured ECs suggest that endothelial healing was not accelerated by succinobucol. In fact, succinobucol may have worsened neointimal formation due to toxicity to ECs, which inhibit SMC proliferation (21;22). Although it has been shown that phenolic antioxidants can provide protection against oxidative stress, they can also lead to generation of secondary radicals that may modify important intracellular targets with the potential to cause pro-oxidant effects (288;289). This may also explain some of the unfavourable cellular responses identified in our study.

In light of the poorly performing SES, one of the most obvious concerns related to possible “overdosing” of the SES on injured arterial tissue. A 1% succinobucol coating was selected for preclinical DES evaluation. This was based on the physical properties of the coating, rather than attempts to predict the coating concentration necessary to replicate previously published cell culture

data. The latter approach is not a common or straightforward method of DES development, since estimating the concentration of drug coating required to achieve a pre-specified local tissue concentration is fraught with difficulties. From prior *in vitro* studies, 10 µM succinobucol exerted potent antioxidant and anti-inflammatory effects, whereas 5 µM was ~50% inhibitory to SMC proliferation (165;166). Unfortunately, quantifying the concentration of drug coating required to obtain this narrow therapeutic range in target tissue would require evaluation of multiple coatings, with repeated measurement of succinobucol levels in the artery wall. The relationship between coating concentration, drug loading and target tissue concentration is dependent on multiple factors including stent surface affinity for drug, *in vivo* release kinetics and subsequent partitioning into local and distant tissues. This level of preparatory work has considerable resource demands, beyond the scope of this research, and its value has not been proven by other groups. Another potential method could utilise complex mathematical modelling, but this prediction is likely to be imprecise as the relationships may not be linear. In my view, *in vitro* techniques to simulate *in vivo* pharmacokinetics are of limited value, not least because most *in vitro* release kinetics are performed in aqueous solutions and the ability to replicate tissue absorption is greatly impaired. Ultimately, 1% succinobucol coating was chosen because it provided excellent coating of stent struts, no overspill onto the balloon and no areas of sparse deposition, which was present with lower concentrations. The local tissue concentration was quantified at various time points after SES implantation and this was compared to in-house cell culture experiments, which were performed *after* the *in vivo* study period, to provide supporting data. The succinobucol concentration measured in arterial

specimens throughout the 28 day study period (200-800 ng/mg, which is equivalent to 300 µM – 1.3 mM succinobucol) was around one hundred-fold greater than the concentration reported to cause antioxidant, anti-inflammatory and antiproliferative effects in previous studies (10 µM succinobucol is approximately 6 ng/mg tissue). However, 1-20 µM succinobucol was found in this study to cause severe toxicity to cells in culture. It was not possible to investigate the effects of much higher concentrations, which were measured in the arterial tissue, to cells in culture due to insufficient solubility. The mass of succinobucol loaded on the SES (~450 µg per stent) was similar to that of other drugs loaded on DES which are even more potent inhibitors of cell proliferation, but share similar release characteristics and tissue solubility. For instance, the tissue concentration of rapamycin measured after successful drug elution in a porcine model (272) was around one thousand-fold greater than that required to inhibit SMC proliferation (281;290), yet this stent is currently used in clinical practice. Similar findings were reported in another preclinical study of polymer-based rapamycin (58), which provided positive data prior to clinical trials. It follows that adjustment of the coating concentration to target a narrow therapeutic range (effective in cell culture) is not a fundamental prerequisite for early DES development. It is clear, however, that drugs may differ considerably in their safety margins. In retrospect, I believe that inclusion of a "low dose" (e.g. 0.1% or 0.5%) and "high dose" (e.g. 1%) SES would have been preferable and this is supported by recently updated consensus guidelines (175).

5.4.2 Effects of rapamycin

The inhibition of neointimal growth by around a third with the RES in this study was very similar to previous preclinical data using an identical non-polymer stent platform (272) subsequently validated in a clinical trial (89), although this finding was not significant. No evidence of medial necrosis was evident in the RES group, but the extent of re-endothelialisation was reduced, albeit non-significantly, consistent with previous reports (62). Inflammation was significantly reduced by the RES, which is in agreement with a similar study of the same stent with rapamycin at a lower concentration (272) and another study evaluating polymer-based rapamycin (58) in a porcine model.

5.4.3 Effects of succinobucol/rapamycin combination

When used in combination, the succinobucol coating impaired the antirestenotic effect of rapamycin. It is likely that the undesirable biological effects of succinobucol on the artery wall opposed the actions of rapamycin, although I cannot rule out the possibility that succinobucol impaired the delivery of rapamycin. In terms of neointimal growth, the dual DES performed similarly to the BMS. Persistent fibrin deposition was present, as in the SES group, indicating suboptimal arterial healing, but inflammation was no different to BMS, suggesting equipoise between the toxic proinflammatory effects of succinobucol against the anti-inflammatory action of rapamycin.

5.4.4 Other predictors of neointimal growth

The increase in EEL area identified in the RES group after 28 days may have been a chance finding. A possible confounding factor was increased rates of

(blinded) implantation of the RES into the RCA, which was a significantly larger vessel than the branches of the left coronary system (mean EEL was 7.86 ± 0.21 mm^2, 7.55 ± 0.23 mm^2 and 8.77 ± 0.35 mm^2 for LAD, LCx and RCA, respectively, $p < 0.05$). This discrepancy in vessel area may have confounded the results for the RES group, although injury scores were well matched. Without baseline measurements, it was not possible to determine the role of positive arterial remodelling, an uncommon sequela of DES implantation, however no cases of incomplete stent apposition occurred (291). Regardless of whether vessel enlargement was acquired or not, neointimal thickening was inversely correlated with EEL ($r = -0.53$, $p < 0.001$), confirming the relationship between smaller vessels and ISR (292). As expected, there was a positive correlation between injury score and neointimal thickening ($r = 0.56$, $p < 0.001$), but importantly, no relationship between vessel size and degree of injury ($r = 0.01$, $p = 0.95$). Injury scores were also closely matched between groups and < 20% of sections showed excessive injury, which is consistent with consensus recommendations (274). Overall the mean injury score was 1.61 (SD 0.19) which was appropriate for the porcine restenosis model.

5.4.5 Premature deaths

Two unexpected premature deaths occurred during the efficacy study. These were probably both caused by acute stent thrombosis. It is recognised that difficulties exist in diagnosing whether stent thrombosis occurred premortem or postmortem. Ventricular fibrillation was witnessed prior to the second death and this was probably the mode of death for the first pig that died suddenly, also within 24 hours. The occurrence of ventricular arrhythmias suggested

unrecognised acute ischaemia, which was most likely due to stent occlusion. It was not possible to determine whether a particular type of stent was responsible for stent thrombosis due to the small number of events. Sudden death may be more common in drug-eluting stents, principally due to stent thrombosis (293), but importantly, the early mortality rate in this study was less than the suggested maximum of 15% for the porcine model (274). To show that succinobucol itself was not likely to be directly responsible for the premature deaths, rather than the stent procedure itself, it was found to be tolerated well when infused down the coronary artery and not found to be proaggregatory (or antiaggregatory) *in vitro*.

5.4.6 Comparison with other studies

In CART-1, although oral succinobucol improved coronary artery dimensions six months after coronary stenting, the drug failed to influence neointimal growth, as observed by intravascular ultrasound (156). The ability of oral probucol to reduce neointimal growth has been variable, with encouraging results in predominantly animal models (148-150;155;262), but negative reports in other studies (153;154;156;157;161;294). Other compounds released from DES have been shown to cause local cell toxicity and increased inflammation following coronary stenting. Polymeric paclitaxel (62;295) and sirolimus-eluting stents (62;272) are both associated with impaired healing, greater inflammation and persistent fibrin deposition after arterial injury. Despite these deleterious effects, both are widely used in clinical practice because of their ability to reduce ISR. Gold-coated stents increased ISR compared with BMS, despite encouraging early preclinical results (296). Aggravated neointimal growth and incomplete healing was caused by an actinomycin-eluting stent (174), as was found in this study. The effects of the

dual SRES contrasted markedly with the effects of a probucol/rapamycin stent on an identical platform, which reduced ISR comparably to a polymeric sirolimus-eluting stent (264). The dual probucol/rapamycin stent also caused no increase in inflammation (263). Therefore, stent-based delivery of succinobucol in combination with rapamycin does not produce similar effects to probucol. This study suggests that a critical factor determining the efficacy of antioxidants used in DES is the balance of their local cellular actions and toxicity. It is also possible that the inferior results of the SRES group compared with the probucol/rapamycin stent may relate to more rapid tissue uptake of succinobucol compared with probucol, which could cause less retardation of rapamycin release from the stent.

5.4.7 Study limitations

This investigation of succinobucol coated stents after arterial injury used a standard preclinical model. It is possible that the effect of local delivery of succinobucol in human atherosclerotic coronary arteries would differ from the effect demonstrated in normal pig coronary arteries. Nevertheless, the morphological characteristics of neointimal growth in the porcine model are similar to that found in human coronary arteries (297). Further study is required to determine the concentration-response (particularly for low dose) and the long-term effects of succinobucol loaded on a stent, although I think a beneficial action is unlikely. The pharmacokinetic profile of the SES was characterised by measurement of drug remaining on the stent at various time points and by estimation of drug levels in surrounding arterial tissue, as described by a recent consensus document for the preclinical assessment of DES (175). *In vitro*

estimation of drug elution was avoided due to the relative insolubility of succinobucol in aqueous solutions. An alternative method to determine drug release involves the use of radio- or fluorescent-labelling to measure the concentration of the succinobucol in blood or arterial segments adjacent to the implanted stent, but this was not possible due to resource demands. Drug concentration was not measured in downstream myocardium from the target artery or in other organs such as liver and kidney to assess the possibility of systemic effects. A very early time point (such as 7 days in the efficacy study) would have been useful to help determine stent thrombosis risk and the presence of early re-endothelialisation. This was not performed in this research to preserve group sizes, although smaller numbers (minimum 3 stents) are probably required for this endpoint. Further characterisation of the inflammatory cells (for instance, semiquantitative division into monocytes/macrophages, lymphocytes and neutrophils) may have helped to unravel the cellular and molecular mechanisms responsible for excessive neointimal growth caused by succinobucol. It would also have been beneficial to section the stents from both premature deaths in the efficacy study, as certain histological features are characteristic of premortem stent thrombosis (variegated platelet-fibrin, thrombus layering and adhesion to the artery wall, and presence of polymorphonuclear leucocytes (274)).

5.4.8 Conclusions

These data suggest that 1% succinobucol is not a favourable stent coating for clinical studies. After 28 days, the SES was associated with widespread inflammation, substantial late fibrin deposition and increased neointimal formation. This maladaptive tissue response is most likely related to localised

cell toxicity, increasing inflammation and impairing healing. The succinobucol coating also inhibited the effects of rapamycin from a polymer-free DES, thereby reducing its antirestenotic properties. More studies are required to determine the role of succinobucol and other antioxidant compounds for the prevention of ISR and promotion of stent healing.

Chapter 6

The relationship between oxidised LDL, endothelial progenitor cells, coronary endothelial function and whole blood aggregation in patients with CHD

6.1 Introduction

Vascular injury caused by PCI elevates oxidative stress (298;299) and leads to increased oxLDL in the blood (114;115), which can exacerbate endothelial dysfunction and impair arterial healing (102;236;300). The mechanisms by which oxLDL exerts these effects are not fully understood. The traditional paradigm of endothelial repair is based on the migration and proliferation of mature ECs from the wound edge to repopulate the site of injury. OxLDL inhibits EC migration (126;235;236) and therefore impairs this process. However, endothelial repair is also now thought to be regulated by mobilisation and homing of bone marrow-derived endothelial progenitor cells (EPCs) to sites of vascular damage, which have the potential to differentiate into ECs (176). Circulating levels of EPCs in blood are increased following several forms of endothelial injury including tissue ischaemia (301), myocardial infarction (302;303) and PCI (304). Mobilisation of EPCs is mediated by cytokines such as VEGF (305) and granulocyte macrophage-colony stimulating factor (GM-CSF) (301), and is thought to be dependent on NO (306). Circulating EPCs are augmented by measures which increase NO bioavailability, such as exercise (307) and drug therapy with statins (308;309) or ACE inhibitors (310). Conversely, the number of EPCs in the vasculature appears to correlate inversely with the presence of cardiovascular risk factors, which reduce NO bioavailability (311;312). Patients with stable CHD have fewer circulating EPCs than healthy controls (311;313;314), and lower levels are associated with worse cardiovascular outcome (315;316).

EPCs are more resistant than mature ECs to the toxic effects of oxidative stress, probably due to greater expression of potent antioxidant enzymes, including manganese SOD, catalase and glutathione peroxidase, which reduce intracellular levels of ROS (317;318). This defence mechanism may allow EPCs to proliferate and differentiate in areas of high oxidative stress, such as inflamed and ischaemic tissues. However, EPC function and survival remain susceptible to oxidative stress (319), and their antioxidant defences may be impaired in the presence of elevated C-reactive protein, a biomarker and active participant in atherosclerosis (125). The addition of oxLDL to cultured EPCs has been shown to impair EPC survival and function in a dose-dependent manner (124;320), an effect attributed to eNOS inhibition (321).

Taken together, the *in vivo* relationship between oxLDL, EPCs and coronary endothelial function is likely to be a critical factor which can influence vessel healing. Oxidative stress, resulting in elevated oxLDL, may increase vascular damage and inhibit endothelial repair due to impaired EPC mobilisation and survival. This could exacerbate coronary endothelial dysfunction. As the endothelium modulates thrombosis and haemostasis, a defect in endothelial function may cause increased blood aggregability, which could increase the risk of stent thrombosis following PCI. A schematic diagram depicting these relationships is shown in Figure 6.1.

Through these suggested interactions, systemic levels of oxidative stress are likely to contribute to the ability of injured arterial tissue to repair after PCI. Oxidised lipoproteins represent one of the major oxidative influences on the

vascular system, being potentially responsible for ongoing endothelial injury, inflammation and impairing healing. There is limited data supporting a direct link between whole blood aggregability and clinical thrombosis (322), however similar methods of platelet testing such as light transmission aggregometry have demonstrated prognostic value (260). The aim of this study was to investigate the *in vivo* relationships between plasma oxLDL levels, the number of circulating EPCs, coronary endothelial function and WBA in patients with symptomatic CHD. This information may provide indirect evidence as to whether elevated levels of oxidative stress (which is increased by PCI) could impair some of the mechanisms involved in arterial repair after stent injury, exacerbating endothelial dysfunction and rendering the stented artery more prone to thrombosis.

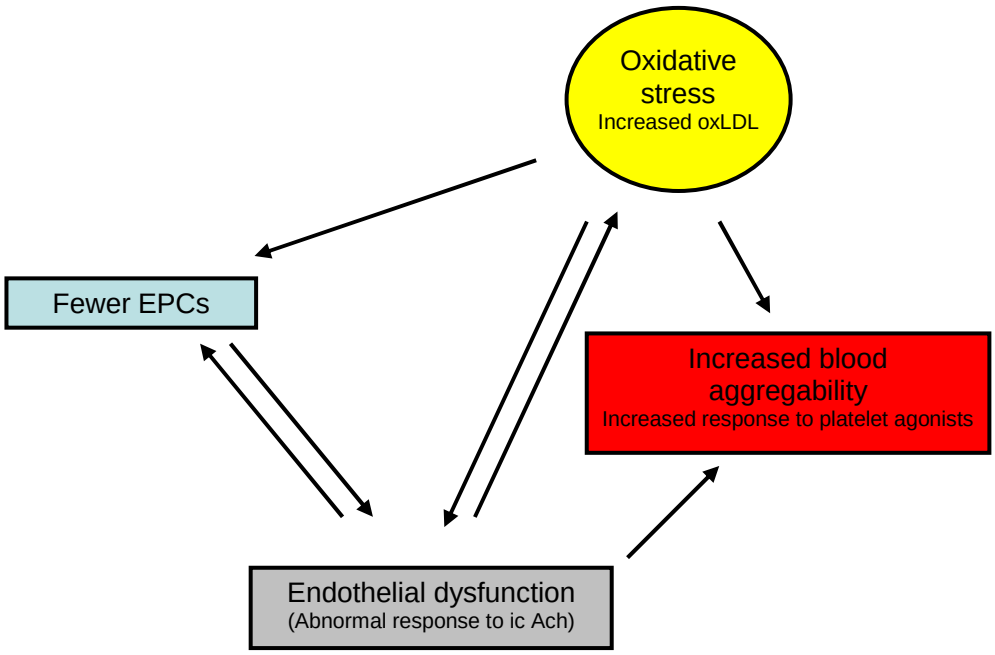

Figure 6.1 The potential relationship between oxidative stress, EPCs, endothelial dysfunction and blood aggregation. Increased oxLDL may lead to fewer circulating EPCs, exacerbating endothelial dysfunction and causing increased blood aggregability.

6.2 Methods

6.2.1 Study population

The study was approved by the West Glasgow ethics committees. All participants were provided with a PIS and gave informed written consent. All patients were scheduled to undergo elective PCI for standard clinical indications. All patients were over age 18 and were receiving oral aspirin and clopidogrel therapy. The characteristics of each patient with respect to factors which can modify endothelial and platelet function were recorded. Patients were excluded if they had a history of myocardial infarction within 3 months, if they were taking any other antithrombotic therapy or if they were unable to give informed consent. On the same day, prior to angiography, venous blood was taken for immediate assay of circulating EPC levels and WBA. Plasma was stored at -70 ºC for subsequent oxLDL assay.

6.2.2 OxLDL Assay

OxLDL was detected by the commercial Mercodia solid two-site enzyme-linked immunoassay (Diagenics, Bletchley, UK). In this assay, two monoclonal antibodies were directed against separate antigenic determinants on the oxidised apolipoprotein B molecule. Samples were snap-thawed at 37 ºC for 3 minutes. All samples were run in duplicate on a single assay. The average coefficient of variation of the duplicates was 0.6%. The lowest sample value was 36 IU, which is above the lowest standard (10 IU). All samples were therefore within the sensitivity of the assay.

6.2.3 EPC Assay

Quantification of EPCs was performed by fluorescent-activated cell sorting (FACS) analysis using a flow cytometer. 1 ml of EDTA anticoagulated blood sample was added to 100 µl of FcR Blocking Reagent (Miltenyi Biotec). After 20 minutes of incubation, 20 µl of the blocked blood was added to 20 µl of anti-VEGF receptor-2 (KDR) antibody with isotype control mouse IgG1-APC used as a control. The blood was incubated for 30 minutes on ice and sheltered from the light. 20 µl of 7-amino-actinomycin D (AAD) viability dye was added for the exclusion of nonviable cells. 20 µl of CD45-FITC/CD34-PE antibody combination was added and CD45-FITC/CTL-PE was used as a control. The solution was incubated at room temperature for 20 minutes, sheltered from the light. Diluted lysis buffer was made up by adding 400 µl 10× buffer to 3.6 ml of water. After 20 minutes incubation, 500 µl of buffer was added before a further 10 minutes of incubation in the dark. 100 µl of fluorospheres was added to prepare the sample for FACS acquisition. Data was analysed using Cellquest™ Pro software (BD). Analytical gates were used to enumerate the total number and subsets of circulating cells. Samples were run until a total of 250,000 events were counted or after a 20 minute cut off. Data were stored for interpretation at the same time by one observer to remove bias. EPC concentration was calculated as: (No. $CD34^{+}KDR^{+}$ events × concentration fluorospheres)/total fluorospheres recovered.

6.2.4 Whole blood aggregometry

WBA was performed according to the method described in Chapter 2.2.3. Collagen (3 µg/ml) and ADP (20 µM) (Labmedics Limited, Manchester, UK)

were used as platelet agonists, within 3 hours of venepuncture. 20 μM ADP is commonly used to assess clopidogrel response (323). WBA was measured as absolute change in impedance 5 minutes after the addition of agonist, compared to baseline.

6.2.5 Coronary endothelial function testing

All vasodilator medication was withheld for 24 hours prior to testing. Coronary endothelial function was measured in a coronary artery without significant stenosis (< 40% diameter), not scheduled for PCI. This was done by the standard method of assessing the change in luminal diameter, using quantitative coronary angiography (QCA), in response to an intracoronary infusion of the endothelium-dependent vasodilator, acetylcholine. In healthy arteries, acetylcholine causes release of NO from ECs, leading to vasodilation. In endothelial dysfunction, the NO response is blunted and the direct muscarinic smooth muscle response to acetylcholine predominates, causing paradoxical vasoconstriction (324). Following baseline coronary angiography, the optimal angiographic projection for the study artery was selected, avoiding overlapping side-branches. A 3 French infusion catheter (Cook Medical, Limerick, Ireland) was placed into the proximal portion of the coronary artery to be studied via a standard 6 French guiding catheter. After intracoronary infusion of 0.9% saline as a control, endothelium-dependent vasomotion was assessed by serial infusions of acetycholine (Miochol-E, Novartis) in the following order: 10^{-6} M, 10^{-5} M and 10^{-4} M. All infusions were given at a flow rate of 2 ml/minute for 2 minutes. Careful attention was paid to the dead space within the infusion equipment to ensure accurate delivery of acetylcholine. Assuming a mean coronary artery

blood flow of 50 ml/minute (325), the final blood concentration for each acetylcholine infusion was estimated at 4×10^{-8} M, 4×10^{-7} M and 4×10^{-6} M, which was appropriate for assessment of a range of vasomotor responses (recommended range 10^{-8} to 10^{-5} M) (326). At the end of each infusion, coronary angiography was recorded using non-ionic contrast medium (Omnipaque™, GE Healthcare) in the standard fashion. Serial angiograms were performed using identical projections, table height and magnification. The acetylcholine infusion was terminated if significant ischaemia or bradycardia were observed. Endothelium-independent vasomotion was then assessed by an intracoronary bolus injection of 400 µg isosorbide dinitrate. If required, a further dose of isosorbide dinitrate was administered to fully reverse any latent effects of acetylcholine, ensuring maximal coronary artery vasodilation, before a final angiogram was recorded.

Quantitative Coronary Angiography

Coronary angiograms were stored digitally at the Golden Jubilee National Hospital, Glasgow for subsequent analysis. An automated edge-detection software system (Centricity CA1000, GE Healthcare) was used to measure the luminal diameter of the coronary artery distal to the infusion catheter, at end diastole. The mean percentage change in luminal diameter compared to control in two distinct 5 mm segments was calculated for each patient following each infusion and after final nitrate injection (each analysis segment consisted of five separate measurements 1 mm apart). Each segment was easily identifiable by anatomical landmarks (such as side branches). Endothelial function was represented by the vasomotor response to the highest tolerated acetylcholine

concentration. One patient did not receive 10^{-4} M acetylcholine due to recurrent transient atrioventricular block. All other patients received all three concentrations of acetycholine. Negative changes in vessel diameter represent vasoconstriction, compared to control. All QCA analysis was performed by a single observer, blinded to all other study results.

6.2.6 Statistical analysis

A formal power calculation was difficult due to the exploratory nature of the study, however a sample size of 23 patients was estimated to provide 80% power to detect a relationship between the independent and the dependent variables at a two-sided 0.05 significance level, if the true change in the dependent variable is 0.3 units per unit change in the independent variable. This was based on the assumption that the SD of the independent variable (oxLDL) is 0.3 and the SD of the dependent variable (endothelial function) is 0.15 (327). A previous small study reported a significant correlation between oxLDL and endothelial response to acetylcholine in 15 patients (328). All data are expressed as mean ± SEM unless otherwise stated. Correlations were performed using the Pearson correlation coefficient. Coronary responses for each infusion were compared using analysis of variance and post-hoc Dunnett's test. Statistical analysis was performed using the SPSS statistical software package 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

6.3 Results

6.3.1 Baseline characteristics of patients

The number of patents included in each part of the study is shown in Figure 6.2. 23 patients consented to the study between June 2007 and April 2010. The endothelial function study was abandoned in one patient due to time constraints in the catheterisation laboratory. All 22 remaining patients underwent coronary endothelial function testing. OxLDL assay was performed in the first 14 patients, but was not available for the remaining patients. WBA assays were performed for all patients. Circulating numbers of EPCs were only available for 18 patients, due to technical problems with the assay. The baseline characteristics of the patients are shown in Table 6.1. All patients had chronic stable angina and were taking standard pharmacotherapy. There was a low prevalence of diabetes mellitus but high prevalence of other cardiovascular risk factors. Most patients had single vessel coronary artery disease.

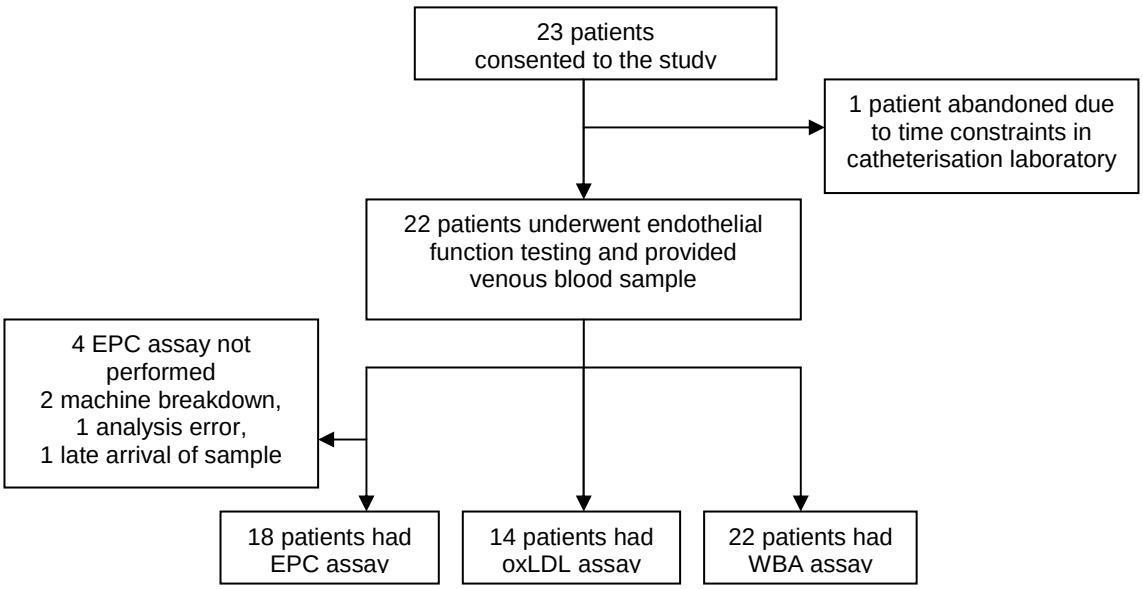

Figure 6.2 Patient flow diagram for study.

Table 6.1 Baseline characteristics of endothelial study patients

	Prevalence, (n = 22)
Patients' characteristics	
Age, mean (SD), y	61.5 (9.5)
Male, n (%)	18 (81.8)
Current smoker, n (%)	2 (9.1)
Hypertension, n (%)	10 (45.5)
Hypercholesterolaemia, n (%)	14 (63.6)
Diabetes mellitus, n (%)	2 (9.1)
Family history of premature CHD, n (%)	9 (40.9)
Previous myocardial infarction, n (%)	7 (31.8)
Previous stroke, n (%)	0 (0)
Previous PCI or CABG, n (%)	0 (0)
Single vessel disease, n (%)	18 (81.8)
Preserved LV function, n (%)	21 (95.5)
Heart failure, n (%)	0 (0)
Drug treatment	
Aspirin, n (%)	22 (100)
Clopidogrel, n (%)	18 (81.8)
Statin, n (%)	21 (95.5)
ACE inhibitor or ARB, n (%)	15 (68.2)
Beta-blocker, n (%)	20 (90.9)
Calcium channel blocker, n (%)	6 (27.3)
Diuretic, n (%)	4 (18.2)
Nitrate, n (%)	10 (45.5)
Nicorandil, n (%)	3 (13.6)

6.3.2 Coronary endothelial function

The mean vessel responses for each acetylcholine infusion and isosorbide dinitrate injection are shown in figure 6.3. The mean response to incremental concentrations of acetylcholine was progressive vasoconstriction, which was significant for the highest concentration (-1.64 ± 1.21%, -4.80 ± 1.84% and -13.20 ± 3.33% for 10^{-6} M, 10^{-5} M and 10^{-4} M acetylcholine, respectively, $p < 0.001$ for 10^{-4} M acetylcholine vs. control). The mean response to isosorbide dinitrate was 11.20 ± 2.50%, $p = 0.001$ vs. control). Figure 6.4 shows the mean endothelial function of two segments for each patient in order of the study performance.

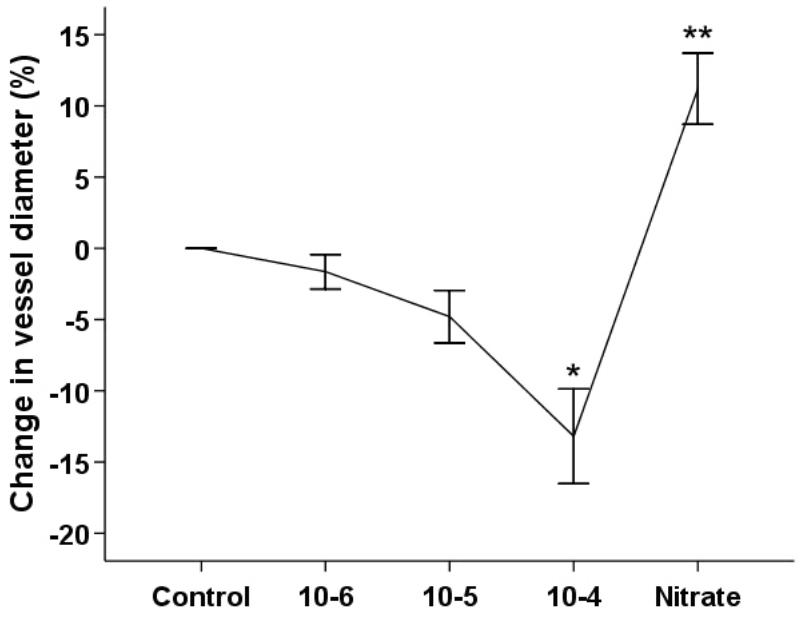

Figure 6.3 Mean vasomotor responses to acetylcholine infusion (concentration [M]) and isosorbide dinitrate. There was progressive vasoconstriction to serial acetylcholine infusions whereas administration of isosorbide dinitrate caused vasodilation (n = 22) * $p < 0.001$ vs. control, ** $p = 0.001$ vs. control.

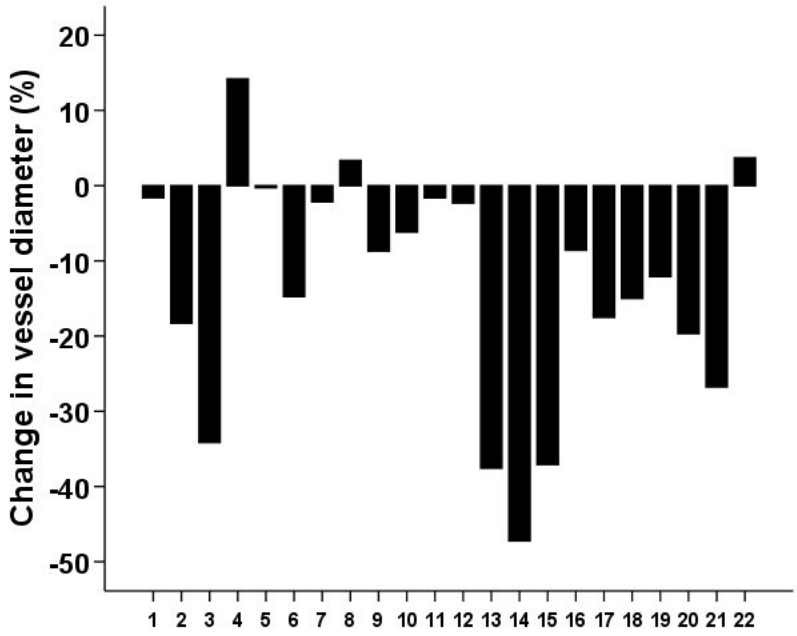

Figure 6.4 Individual patient vasomotor responses to the highest concentration acetylcholine infusion in order of study. The group's mean response was -13.20 (SD 15.60) %.

There was good agreement between the two segments analysed for each patient. The mean difference in endothelial function between the two segments analysed in each patient was 7.87 (SD 8.15) percentage points. 3 patients displayed vasoconstriction in one segment and vasodilation in the other segment.

6.3.3 Adverse events

Transient minor atrioventricular block was common (approximately one quarter of patients) during acetylcholine infusion, especially during the highest concentration, but this was usually short-lived (less than 10 seconds) after the infusion was stopped. Minor electrocardiographic evidence of ischaemia was uncommon and clinical ischaemia was rare (two patients complained of mild angina). Figure 6.5 shows an example of severe vasoconstriction in the left

anterior descending coronary artery in one patient who displayed marked electrocardiographic ischaemic changes during the highest acetylcholine dose, but this was quickly reversed with isosorbide dinitrate injection (serial angiography was successfully recorded). No serious complications were encountered.

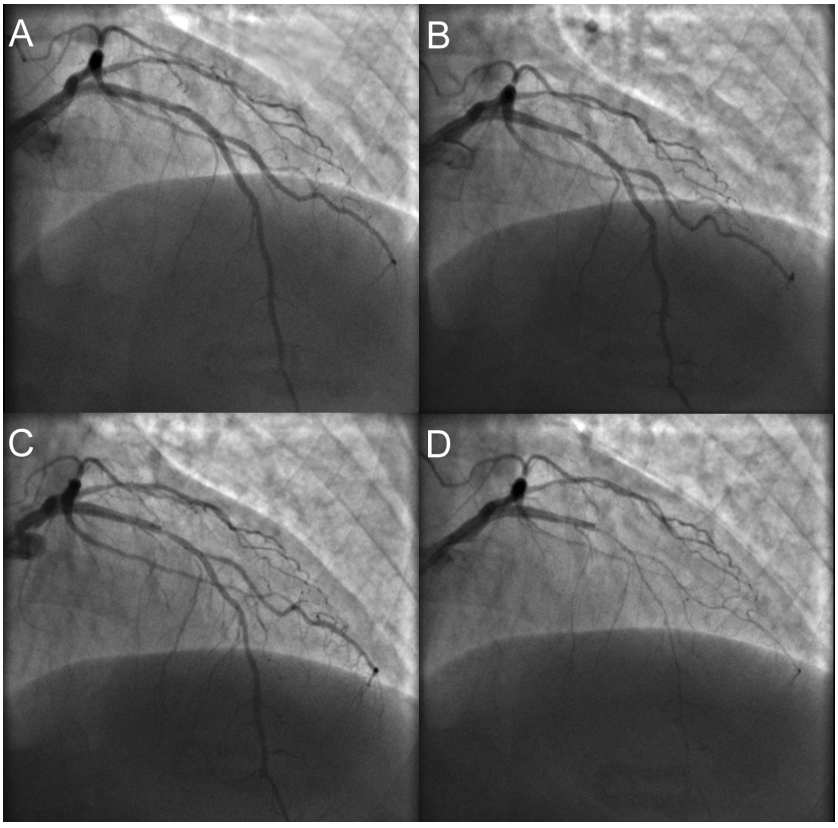

Figure 6.5 Angiographic example of severe coronary endothelial dysfunction. Progressive vasoconstriction of the left anterior descending artery after 0.9% saline (A), 10^{-6} M acetylcholine (B); 10^{-5} M acetylcholine (C) and 10^{-4} M acetylcholine (D) infusions. Isosorbide dinitrate caused complete reversal of vasoconstriction (not shown). All angiograms shown are right anterior oblique 30º cranial projection.

6.3.4 Correlations

Endothelial function correlated significantly with the number of circulating EPCs ($CD34^+/KDR^+$ cells) (Figure 6.6). Patients with fewer circulating EPCs displayed

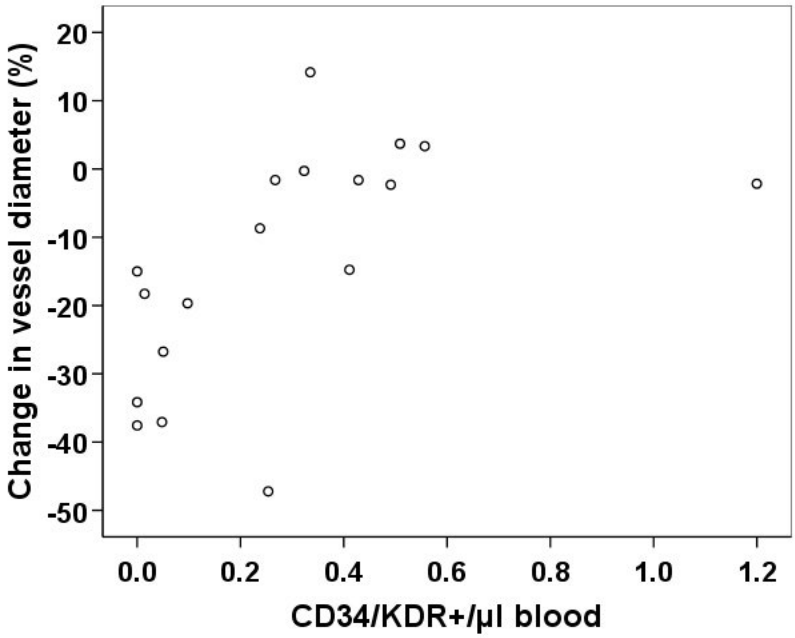

Figure 6.6 Scatter plot of EPC number and coronary endothelial function. Endothelial function is represented by % change in vessel diameter during highest acetylcholine concentration, compared to control. Endothelial function was strongly correlated with EPC number ($CD34^+/KDR^+$ cells) ($r = 0.57$, $p = 0.01$).

more severe endothelial dysfunction. Stepwise multiple linear regression analysis demonstrated that this correlation was independent of age, gender, hypertension, hypercholesterolaemia, diabetes mellitus, current smoking, family history of premature CHD and total number of cardiovascular risk factors ($p < 0.05$). No other significant correlations were found between plasma oxLDL concentration, number of circulating EPCs ($CD34^+/KDR^+$ cells), number of circulating $CD34^+$ cells, endothelial function or WBA, except for a highly significant positive correlation between WBA in response to collagen and WBA in response to ADP (Table 6.2). Figures 6.7 and 6.8 show the lack of correlation between plasma oxLDL and EPC or endothelial function, respectively. Endothelium-independent

vasodilation induced by isosorbide dinitrate injection showed no significant correlation with any parameter (data not shown).

Table 6.2 Correlations between main study parameters.

		oxLDL (IU)	CD34 /µl blood	CD34+/ KDR+/ µl blood	Endo. function	WBA (collagen)	WBA (ADP)
oxLDL (IU)	r	1	-0.134	0.197	0.452	-0.441	-0.369
	p		0.648	0.519	0.105	0.114	0.194
	n	14	14	13	14	14	14
CD34+/µl blood	r	-0.134	1	0.329	-0.089	0.107	-0.294
	p	0.648		0.273	0.763	0.715	0.308
	n	14	14	13	14	14	14
$CD34^+/KDR^+$/µl blood	r	0.197	0.329	1	0.569*	-0.185	-0.040
	p	0.519	0.273		0.014	0.464	0.874
	n	13	13	18	18	18	18
Endo. Function	r	0.452	-0.089	0.569*	1	-0.283	-0.028
	p	0.105	0.763	0.014		0.202	0.903
	n	14	14	18	22	22	22
WBA (collagen)	r	-0.441	0.107	-0.185	-0.283	1	0.692**
	p	0.114	0.715	0.464	0.202		0.000
	n	14	14	18	22	22	22
WBA (ADP)	r	-0.369	-0.294	-0.040	-0.028	0.692**	1
	p	0.194	0.308	0.874	0.903	0.000	
	n	14	14	18	22	22	22

r represents the Pearson correlation coefficient; * $p < 0.05$; ** $p < 0.001$.

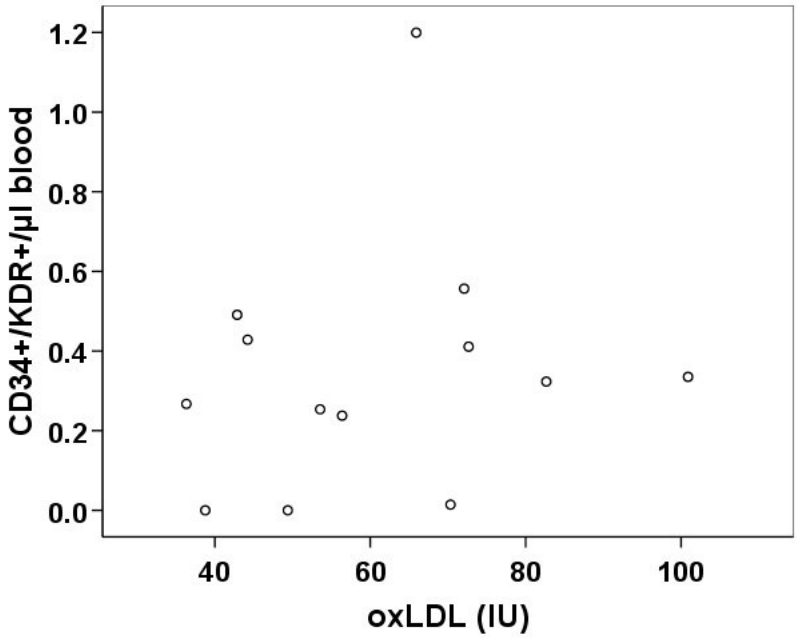

Figure 6.7 Scatter plot of oxLDL and EPC number. There was no significant correlation between oxLDL and EPC number ($CD34^+/KDR^+$ cells) (r = 0.20, p = 0.52).

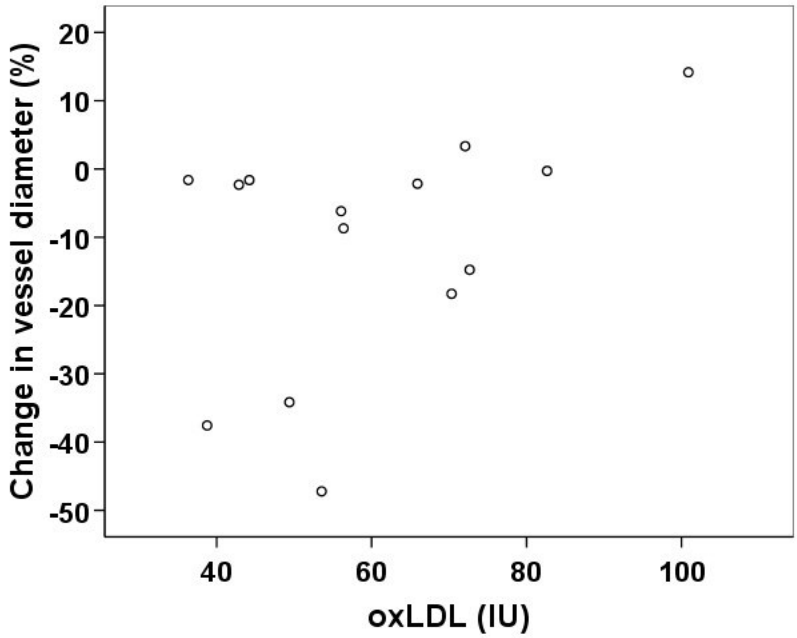

Figure 6.8 Scatter plot of oxLDL and coronary endothelial function. There was no significant correlation between oxLDL and endothelial function (r = 0.45, p = 0.11).

Age was inversely correlated with number of circulating EPCs (Figure 6.9) and positively correlated with WBA in response to 20 µM ADP (Figure 6.10). Stepwise multiple linear regression analysis confirmed that the correlation between age and EPC number was independent of other cardiovascular risk factors ($p = 0.05$), however the correlation between age and WBA in response to ADP was no longer significant after correction for other cardiovascular risk factors ($p = 0.08$). The total number of cardiovascular risk factors (male gender, hypertension, hypercholesterolaemia, diabetes mellitus, current smoking, family history of premature CHD) did not correlate significantly with oxLDL, EPC number, endothelial function or either WBA result (data not shown).

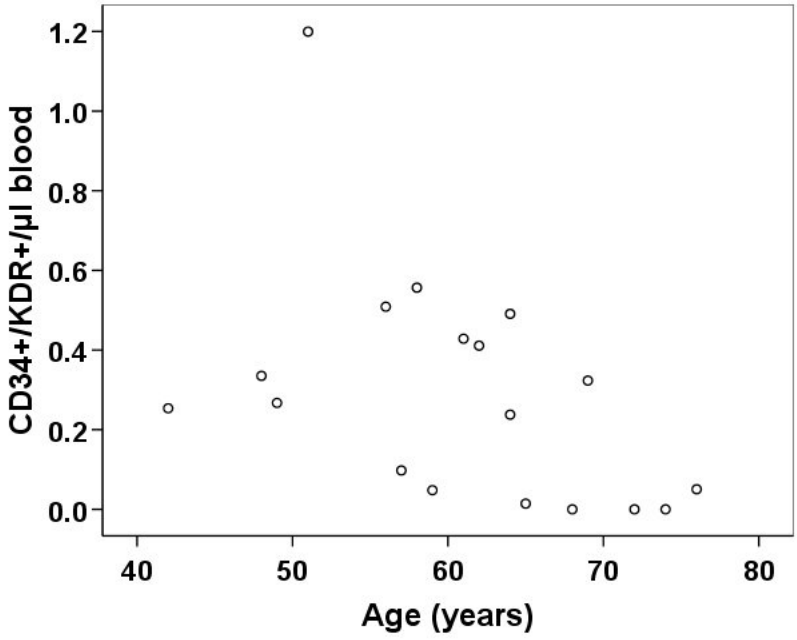

Figure 6.9 Scatter plot of age and EPC number. EPC number ($CD34^+/KDR^+$ cells) was inversely correlated with age ($r = -0.47$, $p = 0.05$).

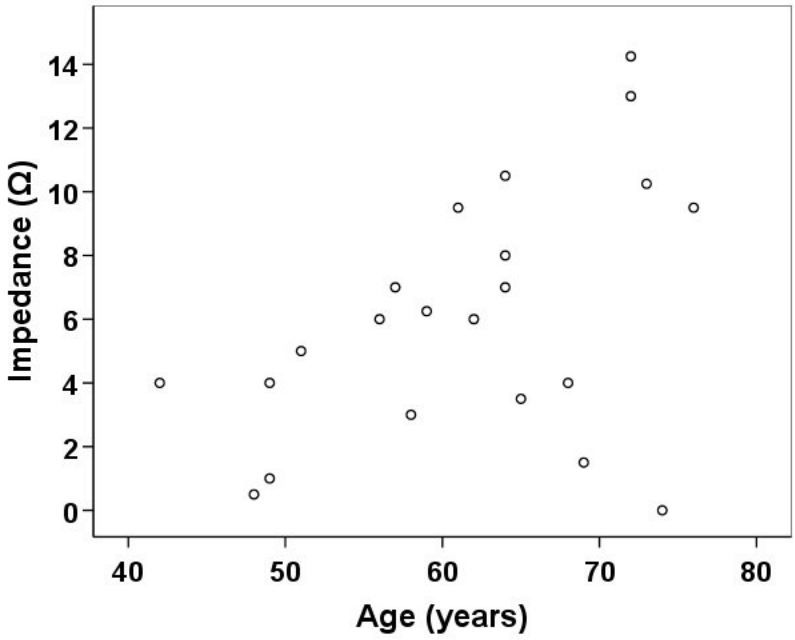

Figure 6.10 Scatter plot of age and WBA in response to ADP. WBA in response to 20 µM ADP was positively correlated with age ($r = 0.46$, $p = 0.03$).

6.4 Discussion

This observational study in patients with stable CHD has demonstrated that coronary endothelial dysfunction was common and correlated with low levels of circulating EPCs. Plasma oxLDL concentration did not correlate significantly with EPCs, coronary endothelial function or WBA.

6.4.1 Endothelial function in stable CHD

The high prevalence of endothelial dysfunction in this population was consistent with other studies in patients with stable angina (324;327;329). Only 1 out of 22 patients displayed significant coronary vasodilation (> 5 % increase in diameter) in response to acetylcholine, suggesting good endothelial function (in that artery). 7 patients exhibited a minimal response to acetylcholine (< 5 % change in diameter). In 14 patients, a definite constrictive response (> 5 % decrease in diameter) to acetylcholine occurred, due to direct muscarinic effects of acetylcholine predominating over acetylcholine-induced NO release from ECs, indicating significant endothelial dysfunction (324;326). These findings reflect the patients' persistent exposure to cardiovascular risk factors and high probability of subclinical atherosclerosis in the study vessel, which cause endothelial dysfunction and reduced NO bioavailability.

The incidence of clinical symptoms during the endothelial function studies was low and consistent with previous work using identical acetylcholine regimens (330). Aiming for a lower estimated final blood acetylcholine concentration

(between 10^{-8} and 10^{-6} M) may reduce the incidence of clinical symptoms further, as in other studies (331).

6.4.2 EPCs and endothelial function

The observation that circulating levels of EPCs in the peripheral blood are correlated with coronary endothelial function is a relatively novel finding. During the initial stages of this research, no studies had reported the relationship between circulating EPC levels and coronary endothelial function.

Hill *et al.* (312) previously showed in 45 individuals without cardiovascular disease that peripheral vascular function (assessed by flow-mediated brachial artery reactivity) was significantly correlated with the number of EC colony forming units in culture, thought at the time to represent EPCs derived from peripheral blood. It has since been established that this widely cited study assessed an aspect of endothelial biology which did not reflect the number of EPCs present in the circulation (332;333). Uncertainty still surrounds the true identity of cells previously described as EPCs in the literature and, crucially, the most accurate way to identify and measure them. EPCs are widely believed to originate from haematopoietic stem cells, which are positive for CD34 (or the more immature marker protein CD133) and the EC antigen, KDR. These putative EPCs appear to make a valuable contribution to vessel formation (334). Hence, the measurement of $CD34^+/KDR^+$ cells is currently thought to be the most appropriate way to define circulating EPCs (333), as was the case in this study. A more detailed discussion on the controversies surrounding the identification and measurement of EPCs can be found elsewhere (176;332).

During the course of this research, Werner *et al.* (335) was the first to report a correlation between EPCs and coronary endothelial function. This landmark study in 90 patients with stable CHD measured the number of circulating EPCs in peripheral blood using flow cytometry to quantify $CD34^+/KDR^+$ and $CD133^+$ cells. The ability of EPCs to produce endothelial colony forming units (described as EPC function) was also measured. It was found that levels of circulating EPCs (using $CD34^+/KDR^+$ and $CD133^+$ methods) and EPC function correlated with coronary endothelial function, assessed invasively using serial acetylcholine infusions. $CD34^+/KDR^+$ cells were more strongly correlated with coronary endothelial function than $CD133^+$ cells. After multivariate analysis, only the number of circulating EPCs correlated with coronary endothelial function independently of traditional cardiovascular risk factors. The failure of endothelial colony forming units to independently predict endothelial function probably relates to their dissociation with the number of circulating $CD34^+/KDR^+$ cells (333) and doubtful function in vascular regeneration (332).

The work in this thesis therefore corroborates the novel findings by Werner *et al.* that EPCs (defined as $CD34^+/KDR^+$ cells) correlate with coronary endothelial function. Evidence is accumulating that suggests EPCs provide an important repair mechanism after vessel damage due to cardiovascular risk factors including hypertension, hyperlipidaemia, smoking and diabetes. Despite this, in many cases, progressive vessel injury progresses to the clinical manifestations of CHD, stroke and peripheral artery disease. The role of EPCs to repair the vessel injury caused by PCI is not well defined, but emerging as an important component of re-endothelialisation and restoration of vessel function (176). By

reducing the capacity of the endothelium to be repaired after vessel injury, an obvious link between circulating EPC numbers and coronary endothelial function exists. The confirmation herein and by Werner *et al.* (335) that lower numbers of EPCs are associated with impaired coronary endothelial function (and hence at risk of subsequent vascular events) provides a basis for the ability of EPCs to predict cardiovascular outcome (315;316). Furthermore, it also provides a valuable link between cardiovascular risk factors and endothelial dysfunction, which are both associated with fewer circulating EPCs.

6.4.3 The role of oxLDL

The relationship between EPCs and endothelial function was independent of traditional cardiovascular risk factors, which implied that EPCs may be causally involved in the regulation of endothelial function and are themselves regulated by other factors. In this study, plasma oxLDL levels were not correlated with the number of circulating EPCs or coronary endothelial function. Therefore, I have failed to demonstrate a biological explanation or peripheral biomarker for the deficiency of EPCs and associated severe coronary endothelial dysfunction in these patients, despite substantial evidence that oxLDL is toxic to EPCs (124;320;321;336-338). Besides the diverse methods to confirm EPC identity in laboratory studies, the absence of correlation in my results may relate to a number of issues.

In previous studies, EPC dysfunction *in vitro* occurred at 1-10 µg/ml oxLDL (124;320) and marginally increased EPC apoptosis (5-10%) was present at 25 µg/ml oxLDL or higher (321;337). Wang *et al.* reported a major reduction in

cultured EPCs (~50%) at 100 µg/ml (336). In my study, the mean (SD) plasma oxLDL concentration was 60.2 (18.3) IU, which was approximately 18.0 (5.5) µg/ml (personal communication from manufacturer), OxLDL levels were therefore lower than would be expected to significantly reduce EPC numbers in the circulation. Furthermore, the studies that investigated statin pre-treatment found that oxLDL-induced EPC dysfunction and senescence were both profoundly inhibited by 1 µM atorvastatin (124;320), possibly via activation of Akt (320;339). Thus, I speculate that the very high prevalence of statin therapy in this contemporary CHD population (90.9%) protected circulating EPCs against oxLDL-induced damage in the blood and may have masked a possible correlation with EPC levels. Further studies could address this relationship in statin-naïve patients; however, blood concentration may represent a blunt way to fully define the interaction between oxLDL and EPCs *in vivo*.

This study was also underpowered to detect minor correlations between oxLDL and EPCs, due to the small number of patients with oxLDL results (n = 14). Nevertheless, there was no signal from the available data that plasma oxLDL was correlated with EPCs. I believe a much larger study would be required to confirm or exclude with confidence a relationship between plasma oxLDL and the number of circulating EPCs. A larger population would reduce the impact of important confounding factors such as co-existing medical conditions and drug treatments, both of which can influence oxLDL and EPC count and probably impaired the statistical validity of this component of my study. If an inverse correlation between oxLDL and EPCs was evident from a larger study, then the ability of oxLDL to predict cardiac events in patients with CHD (340) would

gain further mechanistic insights. Moreover, future directions for novel therapies may be possible, if they could provide EPCs with protection from oxLDL *in vivo* in a similar fashion to statin therapy.

In this study, plasma oxLDL levels also failed to correlate with coronary endothelial function. I believe this relationship was similarly affected by (or possibly even more susceptible to) factors which accounted for the lack of correlation with EPCs; that is, the protective influence of statin therapy, the confounding effects of other risk factors and the relatively small sample size. Hein *et al.* (300) has shown that oxLDL specifically impaired NO-mediated endothelium-dependent vasodilation to shear stress and adenosine, but not bradykinin (a cytochrome *P*-450 monooxygenase activator) in isolated porcine coronary arterioles. A study of 15 CHD patients was first to report an inverse correlation between coronary endothelial function, represented by the vasomotor response to acetylcholine, and plasma oxLDL (328). Importantly, this small study excluded patients taking lipid-lowering therapy, which reinforces the potential role that statins may have played in my results. A larger study in 36 cardiac transplant patients (17% were prescribed statins) demonstrated that coronary endothelial function was inversely related to oxLDL antibody levels, but not circulating oxLDL detected by monoclonal antibody EO6 (341). More recently, Matsumoto *et al.* (342) showed that impaired endothelium-dependent vasodilation in response to bradykinin was weakly correlated with oxLDL levels, measured by an immunosorbent assay.

6.4.4 Other predictors of EPCs and endothelial function

In this study, age was inversely correlated with the number of circulating EPCs. This relationship was independent of traditional cardiovascular risk factors, suggesting that other age-related factors are responsible for the decline in EPCs in older patients. Older patients are likely to be more sedentary than younger patients and this may contribute to the reduction in EPCs, because physical activity increases EPC levels (307), even after a single episode of strenuous exercise (343). Another factor may be the lower use of medications that increase EPC levels, such as statins and ACE inhibitors. In this study, patients prescribed statin therapy were younger than those not prescribed statins (60.2 ± 2.0 years vs. 74.0 ± 2.8 years, respectively, $p < 0.05$). Statin prescription was associated with more circulating EPCs (0.32 ± 0.08 $CD34^+/KDR^+$ cells/µl vs. 0.03 ± 0.03 $CD34^+/KDR^+$ cells/µl, respectively, $p = 0.25$) and improved endothelial function (mean vessel response to acetylcholine -12.4 ± 3.6 % vs. -20.9 ± 5.9 %, $p = 0.33$), but neither result was statistically significant due to the very small number of patients (two) not prescribed a statin. There were no discernable differences in any of these parameters for the prescription of ACE inhibitors. Aging *per se* may be associated with progressive exhaustion of bone marrow derived progenitor cells after several decades of exposure to cardiovascular risk factors and vascular damage (344). Even if not actually reduced in number, a decline in EPC function (345) is likely to make older patients more at risk of endothelial dysfunction and cardiovascular events.

6.4.5 Discussion of WBA results

This study demonstrated that age was correlated with WBA in response to ADP, but not with collagen, in patients taking aspirin and clopidogrel. Since clopidogrel selectively and irreversibly inhibits the ADP receptor on platelets, this suggests a relative lack of clopidogrel response in older patients (346). Indeed, ADP-induced platelet aggregation is probably the gold standard technique to assess clopidogrel response. Variability in clopidogrel responsiveness is well recognised and known to be mediated by numerous clinical, cellular and genetic factors (346). To my knowledge, few studies have specifically reported the influence of age on clopidogrel responsiveness and have provided contrasting results (347;348). Since the correlation between age and ADP-induced WBA was not independent of cardiovascular risk factors, it is likely that other factors such as female gender (an independent predictor after multivariate adjustment in this study), diabetes and smoking are involved (346;348;349).

6.4.6 Limitations

As already discussed, a limitation of this study was the low statistical power to confidently determine the true relationship between plasma oxLDL and other study parameters. However, accurate assessment of these relationships may require a much larger population to avoid the influence of confounding factors. It is not known whether the specific oxLDL assay used in this study may have affected the results, as accurate quantification of oxLDL can be challenging. The method to identify EPCs in this study using specific haematopoietic and endothelial markers, CD34 and KDR respectively, is consistent with

contemporary recommended guidance (332), but the most accurate way to quantify EPCs currently remains speculative (176;333). It would also have been useful to assess EPC function, as this may have revealed an association with plasma oxLDL levels and provided further insights into the high prevalence of severe endothelial dysfunction in CHD patients. It was noted that QCA using automated edge detection software appeared to underestimate the change in lumen diameter for the most severe cases of endothelial dysfunction, compared to qualitative visual assessment, but this was unlikely to have greatly affected the correlations between variables. Despite a statistically significant correlation between EPCs and endothelial function, there was a wide variation in endothelial responses to acetylcholine. This variation is likely to be due to the influence of other mediators which contribute to vascular function, such as the presence of cardiovascular risk factors and other unknown factors. As a result, measurement of EPCs alone is likely to be a fairly blunt predictor of endothelial function in a tested individual.

6.4.7 Conclusions

This observational study has confirmed a high prevalence of coronary endothelial dysfunction in patients with chronic stable angina. Coronary endothelial function in this population was strongly correlated with the number of circulating EPCs, independent of traditional cardiovascular risk factors, which is a relatively novel finding and has only been reported by one other group (335). Plasma oxLDL did not correlate with EPC count or endothelial function, possibly due to the high prevalence of statin use, other confounding variables and insufficient sample size. Future studies should aim to define the specific reasons for a decline in

number and other functional aspects of EPCs in cases of severe endothelial dysfunction. This may aid the development of novel therapies which can protect or accelerate the vital endogenous processes required for rapid endothelial healing after vessel injury, including PCI.

Chapter 7

Concluding discussion

7. 1 Basic concept of the thesis

Oxidative stress plays an important role in vascular repair by influencing multiple targets after arterial injury. Oxidative stress is mediated by numerous ROS including the superoxide anion (O_2^-), which inactivates NO and leads to an accumulation of peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and other potent oxidising secondary molecules. These ROS and others affect critical signalling pathways in platelets and vascular cells after PCI. The principal aspects of this include:

- Stent thrombosis – modulation of platelet aggregation
- Healing – endothelial regrowth and function, EPCs, inflammation
- ISR – re-endothelialisation, neointimal growth, extracellular matrix

The vascular processes modulated by oxidative stress are also closely inter-related themselves. Stent thrombosis is mediated to a large extent by platelets, but it is also influenced by the presence of healthy ECs. The regrowth of ECs not only protects against stent thrombosis, it also restricts neointimal formation, the main cause of ISR. Inflammation is an important element of vascular healing, but it is also strongly related to ISR and can predispose to stent thrombosis. The research presented in this thesis aimed to investigate the effects of oxidative stress on several of these key processes and explore whether a strategy to target ROS would favourably modify vascular remodelling after PCI.

7.2 Oxidative stress: effects on stent thrombosis

7.2.1 Specific effects on thrombosis

Many studies have reported diverse effects of ROS on platelet function and this is discussed in detail in Chapter 2 and by others (177). The overall conclusion from the literature is that ROS are heterogenous regulators of platelet function. Whilst some ROS appear to stimulate platelets, the actions of others may be inhibitory. Stent thrombosis is predominantly made up of platelets, but other factors in the circulation and in the vascular wall are also closely involved, so particular attention in this thesis was given to the effects of ROS on thrombus formation in whole blood and in the vicinity of ECs. The X/XO reaction was used to generate ROS in this thesis and although the formation of specific molecules was not characterised, this reaction is known to produce large amounts of O_2^- and H_2O_2 (238). The effect of oxidative stress in this setting was to inhibit thrombus formation in a concentration-dependent manner. This was an interesting finding, because ROS released by this reaction were expected to cause a stimulatory effect. This early conjecture was fortified by the assumption that ROS were uniformly prothrombotic molecules. By corroborating my results with the extensive literature, it is now clear that this supposition was inaccurate. I speculate that my experiments generated abundant ROS and secondary molecules, which were toxic to platelets and other aggregating factors in the blood. The ultimate effect of this “super-release” of ROS was a decrease in the rate of thrombosis. The addition of ECs to blood also impaired thrombus formation, which was consistent with the primary function of the endothelium. This effect was not significantly altered by the exogenous application of ROS,

suggesting that ROS would not greatly oppose the antithrombotic properties of nearby endothelium after PCI. Consistent with previous work, NO was partly responsible for the inhibitory effect of ECs. Although O_2^- may diminish this influence, the formation of inhibitory ROS appear to counterbalance the effect overall. Of course, whether this experimental situation approximates with the ROS generated by PCI is uncertain. Nevertheless, it would seem that non-specific targeting of the acute effects of ROS on platelets and other aggregating factors in blood after PCI to lower the intrinsic risk of thrombosis may be ineffective. In fact, substantial release of ROS after PCI might even provide some protection against stent thrombosis. The widespread use of intensive antiplatelet medications has dramatically reduced the incidence of stent thrombosis during the first month after PCI to less than 1%, therefore novel approaches to reduce acute thrombotic events further would need to have substantial inhibitory effects on thrombosis. Unravelling the effects of oxidative stress on other vascular processes associated with late stent thrombosis and ISR (such as endothelial regeneration) may offer a more plausable strategy.

7.2.2 Re-endothelialisation

The movement of ECs to regenerate the endothelial monolayer is a critical component of vascular healing. The work presented by this thesis implies that EC migration may be inhibited by ROS, but it may also be antagonised by antioxidants. The explanation for this seemingly paradoxical observation may lie in the fact that pathological release of ROS can clearly disrupt important biological functions but, on the other hand, targeted physiological release of ROS often constitutes an essential component of normal cell activity. In this case, the

impact of ROS is not solely damaging to ECs, but necessary to support motility. The important role of ROS during normal EC migration has been reviewed comprehensively (258). This should guide future strategies away from near-total elimination ROS after PCI, as that may negatively influence some arterial healing processes. The ideal antioxidant to promote endothelial healing would oppose pathological ROS production, but permit lower level physiological ROS release. If this were achieved, antioxidant therapy could potentially accelerate endothelial regeneration. Some data in animal models suggest probucol may be successful for this purpose (149;150), although it would be gratifying if other groups could validate this work. It is not clear whether the reported effectiveness of probucol to promote re-endothelialisation is primarily due to its antioxidant effects or its effects on HO-1 (160;350;351). EPCs may represent another potential target to modify arterial healing after PCI. A major current limitation of this approach is the lack of clear understanding of the identity and precise role of these endothelial precursors. The work in this thesis has confirmed a crucial link between putative circulating EPCs (identified by haematopoietic and EC markers) and coronary endothelial function. This provides further impetus for future studies to fully characterise the role of these cells in vascular repair after PCI and the impact oxidative stress may exert.

7.3 Antioxidants: prevention of ISR

7.3.1 Nitrone D: the good, the bad and the ugly?

The initial compound to be investigated in this thesis was the spin trapping agent, nitrone D, a potent antioxidant, but with relatively few supporting studies. I

found that nitrone D caused slight inhibition of whole blood aggregation. However, subsequent evaluation was abruptly terminated after nitrone D was found to cause a disturbing inhibitory effect on EC migration. Since acceleration of arterial healing was a primary aim of this thesis, there was no option other than to eliminate nitrone D from further investigation. This was disappointing, since nitrone D would have been an intriguing substance to take forward into preclinical assessment. Other than its antioxidant properties, nitrone D has other effects which may have influenced neointimal formation. Nitrone D decreases p21 expression (245), which may be problematic after stent injury because animal studies have indicated that p21 can attenuate neointimal thickening (352). Nitrone D also promotes SIRT 1 transcription, which is a negative regulator of p53 gene expression (245). Although this may be protective towards ECs (249) and EPCs (353), it may accelerate neointimal thickening (354;355). These studies emphasise the need to recognise potentially diverse effects of novel compounds as well as their primary intended action. Until nitrone-based compounds are tested in a preclinical stent model, it is not clear which effects would predominate and which tissue responses that would produce.

7.3.2 Succinobucol: the end of the road?

In contrast to nitrone D, succinobucol had tantalising supportive data which made it an exciting compound to test in a preclinical model. In many respects, the properties of succinobucol matched those of an ideal DES coating. Succinobucol shares many of its appealing properties with probucol, except it is more potent and is taken up more readily by cells (271;356). It also has additional anti-inflammatory effects on vascular adhesion molecules, which are

ideally suited to neointimal suppression. Despite these promising qualities, I found that succinobucol was damaging to arterial tissue after stenting, by increasing inflammation and neointimal growth. These two effects were probably inter-related. Speculation remains as to whether a lower dose would have improved the results. I am not convinced that simply coating a stent with a lower concentration of succinobucol solution would have provided beneficial effects. Based on my pharmacokinetic results, targeting a succinobucol tissue concentration in the range of favourable *in vitro* data (published by Atherogenics, who manufactured succinobucol) would be extremely difficult. Moreover, the cell culture data presented in this thesis suggests that succinobucol at low concentrations is toxic to ECs and SMCs. Of course, toxicity towards SMCs is not necessarily a negative characteristic of a DES, as this may limit neointimal proliferation, but the risk of inflammation and impaired healing would present a similar situation to that already offered by current DES, which are also uniformly toxic to vascular cells. Incidentally, the development of succinobucol as an oral medication to treat cardiovascular disease was suspended during the writing of this thesis after disappointing results in clinical trials.

7.4 The antioxidant-eluting stent: still a worthwhile concept?

This thesis investigated two novel antioxidant compounds, with ultimately negative effects. In fact, neither came close to providing promising data. In many respects, this contradicts the scientific literature that has broadly endorsed the potential role of antioxidants in vascular remodelling after PCI. The relevant studies have been reviewed in Chapter 1. Animal studies of probucol have

yielded the most positive results, yet this drug has not been effective in humans. Clinical studies of oral probucol which included IVUS measurements have consistently found that this drug has no effect on NIH (156;157;161). Oral succinobucol was identical in this regard (156). It was a central hypothesis of this thesis that local delivery of antioxidants would circumvent the obvious limitations of oral dosing in humans compared to animals, but no studies have reported a positive result for this strategy. Incorporation into dual DES technology may offer the only viable alternative. It is worth noting that successful reports of a probucol/rapamycin-eluting stent have conspicuously omitted probucol as a sole coating (264). The efficacy of a probucol- or succinobucol-eluting stent therefore seems improbable, so attention should probably be re-focussed on other antioxidant techniques.

The obvious question relates to which antioxidant therapy holds most promise for human applications. Given that NADPH oxidase is the principal contributor to ROS production after PCI, it is conceivable that inhibitors of this enzyme might offer the greatest chance of success (143;144). Antioxidants that decompose O_2^- and H_2O_2 (both promote SMC proliferation) may be a worthwhile strategy and several animal studies using SOD with and without catalase have reported positive results (145;146;357-360). Unfortunately, the targeted delivery of these agents in humans provides significant challenges. Their ultra-short half-life in the circulation presents their foremost limitation: the requirement for viral gene transfer. Viral transfer of eNOS (361) and VEGF (29) has been effective in animal models but suffers from the same disadvantage, which is significant. The concept of a gene-eluting stent has not been successfully translated to humans.

Accumulation of viral vectors in non-target tissues is a concern, given their relatively narrow therapeutic index and potentially harmful immune responses. Nevertheless, whilst gene-based therapy in general for cardiovascular diseases has so far provided limited successes, it offers the potential to make a major impact if translated to humans successfully. *Ex vivo* antioxidant gene transfer of progenitor cells may provide an alluring prospect in the future (362).

Ultimately, will an antioxidant-based strategy ever be successful in improving clinical outcome after PCI? Innumerable animal models have provided satisfying results, whereas human studies have been universally disappointing. This situation unfortunately mirrors the use of antioxidants in cardiovascular disease in general. It is difficult to envisage that antioxidants will ever induce favourable vascular responses after PCI to rival the success of established devices. The arrival of new generation DES on the market will elevate the competition further. An antioxidant-based approach would need to be potent enough to inhibit profound tissue stimuli without causing toxicity. At the same time, it must permit ROS signalling which is necessary for normal vascular cell function. These challenges may prove insurmountable for an antioxidant, but future attempts are awaited with great interest.

7.5 Final conclusions

In conclusion, this thesis has examined the role of oxidative stress in platelet and EC function, known be important after PCI, and found this to be damaging. Despite this, the effects of a novel antioxidant-eluting stent were detrimental to

vascular repair. Coronary endothelial dysfunction was correlated with reduced circulating EPCs, but plasma oxLDL was not related to either parameter. These findings highlight the difficulties and complexities of targeting oxidative stress to improve outcome after PCI. I hope the work contained in this thesis will stimulate clinicians and scientists alike to continue with research in this exciting and rapidly progressing area of cardiovascular disease.

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