Techniques to Preserve Endothelial Cells in Vein Grafts

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Endothelial cells comprise the intimal layer of the vasculature, playing a crucial role in facilitating and regulating aspects such nutrient transport, vascular homeostasis, and inflammatory response. Endothelial dysfunction is believed to be a key driver for vein graft disease—a pathology in which vein grafts utilised in coronary artery bypass graft surgery develop intimal hyperplasia and accelerated atherosclerosis, resulting in poor long-term patency rates. Activation and denudation of the endothelium following surgical trauma and implantation of the graft encourage a host of immune, inflammatory, and cellular differentiation responses that risk driving the graft to failure. Several approaches have been developed to mitigate the onset and progression of this pathology both clincally and surgically, including optimisation of surgical technique, vein preservation conditions and pharma-modulation. Novel approaches are also under investigation in recent years, including the use of topical gene therapy and the utilisation of endothelial progenitor/colony-forming cells to regenerate vein grafts with the view to improving patient outcomes.

Keywords: endothelial dysfunction ; vein graft disease ; intimal hyperplasia

1. Harvesting Techniques

The conventional technique (CT) for vein harvest as initially described by Favaloro et al. [1] entails vein exposure along its length through a longitudinal incision following the contours of the leg and is then harvested in a skeletonised fashion, stripped of surrounding peri-vascular tissue. This induces a degree of venospasm that is subsequently counteracted with manual distention of the vein using crystalloid or blood solutions. Moreover, inflating the vein during the harvest to check for side branches at pressures significantly greater than that of the human vascular system disrupts the endothelium [2], promotes thrombosis [3] and damages the tunica within the vein wall [4]. Damage to the endothelium alters the release of endothelial-derived vasoactive substances including NO, prostanoids, and endothelin-1. In response to damage, the EC production of NO and prostacyclin is reduced but production of prothrombotic factors such as thromboxane and endothelin-1 is increased [5]. Damaged endothelium is pro-coagulant and acute thrombosis is a primary cause of early graft failure [6].

The no-touch technique (NTT) was first described by de Souza in 1996 [7]. Souza proposed that the vein could be harvested with surrounding adventitia, and by doing so, the vein is cushioned by a border of soft tissue which contains the important perivascular structures such as vasa vasorum (VV) and nerves. Importantly, the vein is not directly handled during this process. This technique preserves both the endothelium of the vessel and the microvasculature that nourishes the vein wall. The VV has been shown to maintain perfusion of the vein wall through direct contact with the vein lumen after harvest [8] and demonstrate much greater intimal density and penetration compared to arterial grafts, suggesting a greater significance in the maintenance of luminal viability after harvest. Stripping of the VV by CT therefore promotes ischaemia within the LSV wall with a subsequent detrimental impact on patency, [9] which is likely of larger impact than the associated ischaemia of arteries harvested by CT. The NTT therefore avoids physical disruption to these surrounding tissues, limiting ischaemic injury to the endothelium and provides a physical buffer to limit inadvertent kinking of the graft.

Most of the surrounding ‘cushion’ with which the vein is harvested is composed of fat. Lipocytes have been demonstrated to be a vital source of locally derived vasoactive factors essential for the regulation of vascular tone [10], especially NO. There is compelling evidence that NO expression in vein grafts after CABG plays a key role in the reduction of both IH and atherosclerosis in mouse models [11]. Dashwood et al. [12] demonstrated the presence of eNOS and NO within the periadventitial fat surrounding the LSV following harvest using NTT. This suggests that another benefit of the NTT is not only avoidance of direct physical insults, but also by preservation of the vasoactive sources local to the vein. A further benefit of the NTT relates to the minimised distention of the vein during preparation, which is known to impact on EC integrity, and was previously demonstrated by Angelini et al. [4][13] who confirmed previously that elevated distention pressure causes a reduction in the concentration of adenosine triphosphate (ATP) within the vein and that preservation fluids have the potential to improve ATP levels. The superiority of the NTT was more recently confirmed clinically with

higher patency rates of veins harvested by this method as compared to radial arteries or veins harvested conventionally. Furthermore, this also appears to translate into better outcomes as demonstrated by Tian et al. [14] in their multicentre randomised control trial of 2533 patients. It is essential to note that the NTT is more challenging in patients who are obese and when the LSV is very superficial due to anatomical variation [7].

2. Storage Conditions

Once harvested, the LSV is stored in a preservation solution of the surgeon's choice whilst the heart is prepared for grafting. The solution of choice is a spectrum of crystalloid to autologous blood solutions with various additives intended to buffer pH, confer osmotic impact, act as antioxidants, and mimic the normal composition of bodily intracellular fluid. During this time spent following harvest and prior to implantation, the LSV is in a period of relative ischaemia. The role of preservation fluids is therefore to attenuate EC damage where possible [15]. The primary source of ATP within the LSV is SMCs within the vein media and so the metabolic function of SMCs can be considered by the available concentration of ATP. Heparinised autologous whole blood (AWB) has been shown to better preserve ATP levels compared to normal saline. In fact, AWB has been shown in several studies to improve the vascular contractile reserve and EC function compared to normal saline [15][16][17] through maintenance of SMC tone and integral EC functions such as vasoactivity and platelet activation. Whilst heparin itself exhibits toxicity to the endothelium [18], the addition of whole blood appears to be protective which may be a consequence of its natural contents of energy sources, pH buffers, free-radical scavengers [16].

Papaverine is a commonly evaluated component of preservation fluid to induce SMC relaxation [19]. It is suggested that it may cause chemical damage to the endothelium due to its acidic pH and may also reduce local prostacyclin production. However, it has been demonstrated to reduce venospasm and EC preservation compared to AWB or isotonic crystalloids alone. The vein wall reacts to most local stimuli by undergoing prolonged muscular contraction, which disrupts the endothelium by reducing luminal surface area and causing herniation of SMCs into the lumen which, in turn, denudates the EC layer [20]. Increased SMC relaxation by vasodilators such as papaverine therefore mitigates venospasm, allowing the use of lower distention pressures during harvesting, thereby attenuating loss of ECs and EC damage.

More recently, new solutions have come to market. DuraGraft® (Somahlution, Jupiter, FL, USA) is marketed as a one-time intraoperative treatment for the purposes of reducing vascular EC damage. It is a physiological salt solution (PSS) with additives to attenuate ischaemia and reperfusion injury [21]. Although, theoretically, solutions like these can make a difference, there is very limited data available to compare the efficacy of such solutions. A prospective ex vivo analysis [22] of 26 LSV segments (2 per patient) of which half (n = 13) had been preserved with DuraGraft® and the other half with heparinised Ringer's lactate for ≥90 min. Veins treated with DuraGraft® demonstrated greater CD31 staining ($p < 0.05$) and reduced intimal swelling. There was also greater cell viability as demonstrated by Ki67 staining ($p < 0.01$) and analysis of cell death and senescence marker γH2AX demonstrated reduced numbers of arrested cells or cells with DNA damage in the EC layer of the DuraGraft® group ($p < 0.05$). Furthermore, using phosphorylated-p53 as a marker of apoptosis, there was a significant reduction of cell death in veins treated with DuraGraft® compared to Ringer's lactate. Features of hypoxic stress were identified more frequently in non-DuraGraft® treated conduits, although there was no clear reduction of ROS in this group. This study suggests that DuraGraft® attenuates hypoxic injury and better preserves endothelium compared to Ringer's lactate. Additionally, a comparison of PPS, AWB and DuraGraft® to evaluate endothelial integrity of venous segments was undertaken by Toto et al. [23], which identified a statistically significant reduction in apoptotic cells in the DuraGraft® storage sample after 2 h incubation versus PPS, with apoptotic cells identified using DNA fragmentation detection with fluorescein-12-dUTP. A further evaluation of DuraGraft® by Tekin et al. [24] showed significantly lower total oxidative status (i.e., total oxidant molecules present) for DuraGraft® stored samples, compared to both saline and AWB ($p < 0.0001$). The total antioxidant status (i.e., the ability to neutralise ROS) was lowest in the saline group and equal between both AWB and DuraGraft®. Combined, these findings suggest that the veins stored in DuraGraft® had higher capacity to combat oxidative stress from ischaemia and therefore better protect EC function.

3. Pharma-Modulation

Pharmacological intervention plays a key role in secondary prevention of vein graft failure. Antiplatelet therapy has played a crucial role in improving outcomes after CABG and reducing vein graft failure, with administration of antiplatelets such as aspirin frequently utilised in clinical practice to mitigate complications following surgery [25].

3.1. Aspirin

The long-term benefit of aspirin after CABG is mediated through reduction and prevention of platelet aggregation and a resultant reduction of thrombus formation, largely mediated by a reduction of thromboxane A2 (TXA2). Beyond platelet

inhibition, aspirin also has a significant role in upregulation of EC NO synthesis [26]. The beneficial cardiovascular effects of aspirin are mediated through its inhibition of cyclooxygenase–1 (COX-1) activity. COX-1 is expressed within ECs and is responsible for the production of prostaglandins and resultant production of prostanoids such as TXA2. TXA2 stimulates platelet aggregation and the release of vasoactive pro-coagulant and pro-inflammatory factors; thus, many of the favourable effects of aspirin are attributed to the inhibition of TXA2. TXA2 has been shown to interfere with key endothelium-dependent pathways responsible for regulating blood flow via modulation of calcium activated potassium channels and impairment of cell signalling between EC and SMC layers, thereby impairing EC dependent vasodilation [27]. Aspirin has also been shown to evoke activation of eNOS and therefore increase NO synthesis. This occurs not only within ECs but within platelets themselves and appears to be independent of both COX-1 inhibition and TXA2 production. Two randomised studies have shown a significant increase in markers of NO formation (i.e., HO-1 and ADMA) in a non-dose-dependent fashion in patients with cardiovascular disease, supporting the hypothesis that an aspirin-mediated benefit occurs through the formation of NO, not just the commonly reported mechanism of platelet inhibition [28][29].

3.2. Statins

Statins are predominantly utilised for their lipid-lowering properties. However, when given perioperatively to patients undergoing CABG, they have been shown to improve EC function, maintain NO levels and promote antioxidant activity, as well as inhibiting vasoconstriction, thrombosis, and the inflammatory response. Yang et al. [30] were able to demonstrate direct enhancement of saphenous vein EC expression of eNOS and subsequent increased NO production from ECs in response to statin therapy. A reduction in low-density lipoprotein (LDL) cholesterol has also been shown to result in reversal of EC impairment [31]. The clinical associations have also been proven—one of the first studies to randomise patients to statins after CABG was able to demonstrate that aggressive statin treatment (40 mg/day) after CABG was associated with less angiographic evidence of vein graft occlusion. Specifically, they identified an average of 10% increased vessel occlusion in patients undergoing aggressive statin therapy versus 21% increased occlusion in patients undergoing moderate (2.5 mg/day) statin therapy ($p < 0.0001$), as well as a reduced mean number of grafts exhibiting progression of atherosclerosis (25% in the aggressive treated group versus 39% in the moderate treated group, $p < 0.001$) [32].

3.3. ACE Inhibitors/AngII Receptor Antagonists

Angiotensin-converting enzyme (ACE) is a key regulator of the renin angiotensin aldosterone system. Renin, which is synthesised as an inactive pre-pro-hormone, undergoes a proteolytic cascade resulting in its release into the system circulation as its active form. Within the circulation it acts upon angiotensinogen to generate angiotensin-I (AngI). AngI is subsequently cleaved by ACE to produce AngII. AngII is the primary atherogenic effector of the renin angiotensin aldosterone system, and activation of this system is associated with increased atherothrombotic events [33].

Many in vitro and in vivo animal studies have implicated AngII within pathways known to contribute to vein graft disease. AngII has been found to promote IH [34] and both SMC hypertrophy [35] and proliferation [36][37], and is evidenced to activate key inflammatory pathways which precipitate vein graft disease. Specifically, AngII has been demonstrated to stimulate release of IL6 from SMCs and macrophages [38] and to activate nicotinamide dinucleotide phosphatase oxidase with resultant production of ROS [39][40], thereby promoting oxidative stress within the vascular microenvironment. Angiotensin-converting-enzyme inhibitor (ACEi), Ramipril, assists in the preservation of EC function by inhibiting AngII production and attenuating the aforementioned pathways promoting inflammation and oxidative stress [41]. They also enhance NO production through prolongation of the half-life of bradykinin and stabilisation of the bradykinin receptor linked to the formation of NO [42]. Similarly, AngII type-1 receptor antagonists (ARBs) (i.e., losartan) exert similar benefit through competitive inhibition of AngII via its receptor.

Several human studies have evidenced improved arterial EC function following the administration of ACEi and ARBs in patients with diabetes [43] and hypercholesterolaemia [44]. Several large randomised studies including QUO VADIS [45] and IMAGINE [46] have looked at their clinical benefit after CABG but did not assess features of EC damage or post-operative vein graft patency directly. A 2005 blinded, randomised trial by Trevelyan et al. [47] demonstrated improvement in systemic endothelial function following pre-operative patient treatment with both ACEi (enalapril) or ARB (losartan). EC function, quantified by endothelial dependent flow mediated dilatation (FMD) of the brachial artery, increased from baseline in all groups (5.2% in surgery and enalapril group, $p = 0.015$ versus 5.0% in surgery and losartan group $p = 0.0004$ versus 3.0% in surgery alone group $p = 0.05$) at three months after CABG and was sustained at five months post-operatively. Interestingly all patients, even those who did not receive any pharma-modulation, demonstrated improved systemic endothelial function after CABG suggesting that coronary revascularisation alone confers some improvement of endothelial function.

Furthermore, research into lectin-like oxidised-LDL receptor-1 (LOX-1) has been shown to increase in response to ox-LDL and specifically in atherosclerotic plaques (such as those found in vein graft disease) [48][49][50]. These receptors have also been shown to upregulate in response to AngII through activation of AngII type-1 receptors, and interestingly the use of the ARB losartan inhibits LOX-1 upregulation [51][52][53]. Work by Ge et al. [54] utilising rabbit models identified that losartan treatment attenuated lesions and improved plaque stability; however, no reduction in intimal area was observed compared to the untreated group. LOX-1 expression was shown to increase in both the endothelium and lesion area of control mice, which appeared to be attenuated upon losartan treatment, suggesting that LOX-1 downregulation may confer benefits in the attenuation of vein graft disease.

4. Novel Approaches to Endothelial Preservation

Beyond the aforementioned approaches to endothelial preservation, several novel approaches are being considered including, but not limited to, gene therapy and utilisation of endothelial progenitor/colony-forming cells (EPCs/ECFCs).

Many of the most recent advances in gene therapy relating to vein graft disease have been reviewed in detail by Southerland et al. [55]. In brief, vein graft disease presents as a strong candidate for this treatment approach, predominantly due to the ability to administer the therapy ex vivo prior to reimplantation into the arterial circulation. This approach has the effect of ensuring localised delivery of the therapeutic whilst mitigating the effects of off-target or systemic effects.

There have been a significant number of pre-clinical studies undertaken to investigate the feasibility of gene therapy, with the primary targets relating to endothelial and smooth muscle cell preservation, and mitigation of thrombosis and inflammation. Approaches have included intraluminal or pressure-mediated transfection to induce eNOS overexpression [55][56], COX-1 overexpression [57][58], thrombomodulin and tPA overexpression [59][60], MCP1 inhibition [61][62], NF-κB inhibition [63][64], and E2F inhibition [65] among others. Of note, the E2F inhibition approach via oligodeoxynucleotide delivery had been expanded to human subjects (PREVENT trial) following success in pre-clinical models; however, outcomes of phase 3 have not shown significant differences in graft stenosis or patency by 12 months.

As an alternative approach, researchers are also investigating the potential of endothelial progenitor/colony-forming cells as a means of facilitating re-re-endothelialisation of saphenous vein grafts following implantation, with much of this branch of research having been concisely summarised by Paschalaki et al. [66]. In brief, EPCs categorise numerous populations of cells which express markers endothelial-specific markers which are known to contribute to vascularisation. ECFCs, as a subset, have been identified as having characteristics specific to endothelial origins, and these cells have been investigated in relation to a substantial number of vascular diseases, and have been considered in clinical applications such as gene therapy, vessel/tissue bioengineering, endothelial preservation, and repair.

With specific consideration of vein graft disease, circulating EPCs have been shown to regenerate graft endothelium in mice models, with approximately one-third of regenerated ECs derived from this circulating, bone-marrow derived population [67]. Furthermore, phenotypic analysis of peripheral blood derived ECFCs, compared to both human-derived arterial and venous ECs under high pulsatile flow conditions, depicted a highly proliferative, adaptable cell population which conforms to hemodynamic conditions, adopting a phenotype resembling that of the arterial ECs [68]. Interestingly, a study conducted by Feng et al. [69] was able to identify a therapeutic approach to increasing incorporation of ECFCs into vein graft endothelium through the topical treatment of high-density lipoprotein to the vessel adventitia. This treatment approach in mice showed a significant reduction in neointimal area ($p < 0.001$), improved blood flow ($p < 0.0001$), reduced inflammatory response ($p < 0.05$) determined by leukocyte adhesion, and enhanced endothelial regeneration ($p < 0.05$). This regeneration was attributed to improved ECFC migration and adhesion, which appear dependent on scavenger receptor class B type 1 expression, extracellular signal-regulated kinases and NO signalling.

Another study of note utilised human umbilical cord blood endothelial progenitor cells as a means of facilitating re-endothelialisation of vein grafts [70]. This group were able to identify that these cells exhibit superior adhesion capacity to cultured SMCs under shear stress conditions (0.5 dyne/cm^2) compared to arterial ECs and peripheral blood ECs, believed to be the result of increased expression of cell surface $\alpha_5\beta_1$ integrin. The proliferative capacity of these cells, to facilitate potential re-endothelialisation was also shown to be significantly faster under both shear conditions (0–15 dynes/cm^2) and compared to arterial and peripheral blood ECs. Finally, using an immunodeficient vein graft mouse model, the group identified, with results indicating that these progenitor cells were capable of accelerating graft re-endothelialisation and, consequently, mitigating graft thrombosis. Whilst these results are promising, the haemodynamic conditions assessed here are significantly lower than those of the in situ arterial environment, and the highly thrombotic mouse model likely

overestimates the anti-thrombotic nature of these cells. However, despite these drawbacks, EPCs present as a novel therapeutic approach to the preservation of endothelial function.

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