Perivascular paclitaxel wraps block arteriovenous graft stenosis in a pig model

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Abstract

Background. Haemodialysis vascular access dysfunction is currently a huge clinical problem. In an attempt to reduce the morbidity associated with haemodialysis vascular access dysfunction, we have previously developed and validated a local perivascular paclitaxel release system that has been shown to release paclitaxel for at least 3 weeks. The aim of the current study was to evaluate the in vivo use of these perivascular wraps (for both safety and efficacy) at different time points in our pig model of arteriovenous graft stenosis.

Methods. Paclitaxel-loaded ethylene vinyl acetate wraps were placed around the graft-vein anastomosis on one side, with control polymers being placed on the contralateral side in our pig model of arteriovenous graft stenosis. Animals were sacrificed at early (10–11 days), middle (23–24 days) and late (32–38 days) time points. The entire graft-vein anastomosis was removed at the time of sacrifice and assessed for the extent of luminal stenosis using histomorphometric techniques.

Result. Graft-vein anastomoses treated with the paclitaxel-loaded polymers had an almost complete absence of luminal stenosis at the middle (23–24 days) and late (32–38 days) time points (when one would expect the development of neointimal hyperplasia) as compared with the contralateral control graft-vein anastomoses (37.90% luminal stenosis in the controls vs 0.10% in the paclitaxel group). There were minimal local side effects from this procedure.

Conclusions. Our results demonstrate the safety and efficacy of paclitaxel-loaded perivascular wraps in the setting of a pig model of arteriovenous graft stenosis. We believe that such a local approach which could be easily applied at the time of surgery is ideally suited for use in the clinical setting of haemodialysis vascular access dysfunction. It is likely that this novel approach could result in a significant reduction in the huge economic and health morbidity costs currently associated with this recalcitrant clinical problem.

Keywords: anti-proliferative therapy; dialysis access stenosis; haemodialysis vascular access dysfunction; local drug delivery; neointimal hyperplasia; perivascular drug delivery

Introduction

Haemodialysis vascular access dysfunction is a major cause of morbidity and hospitalization in ~300,000 patients currently on haemodialysis in the US, at an economic cost of ~1 billion dollars per annum or ~8000 dollars per patient at risk per year [1–3]. The main cause of haemodialysis vascular access dysfunction is the occurrence of venous stenosis in arteriovenous dialysis access grafts and fistulae (especially in the former), which ultimately results in thrombosis of the dialysis access [4–7]. We and others have previously demonstrated that at a histological level, venous stenosis in dialysis access grafts and fistulae is due to venous neointimal hyperplasia (VNH) which is comprised of smooth muscle cells, fibroblasts and myofibroblasts, microvessel formation within the neointima and the presence of a perigraft macrophage layer [4,8]. Despite the magnitude of the clinical problem, however, there are currently no effective therapeutic interventions for haemodialysis vascular access dysfunction [4].

Paclitaxel is an anti-proliferative chemotherapeutic agent which results in a polymerization of microtubules within the cell and thus results in a G2/M cell cycle arrest. Although extremely effective at blocking the proliferation of a variety of malignant cell lines and tumours, the significant clinical toxicity of paclitaxel has negated its systemic administration for clinical...
conditions characterized by non-neoplastic tissue growth, such as neointimal hyperplasia NH and vascular stenosis. The local administration of paclitaxel directly to the site of injury, could however, allow the effective use of this agent with minimal systemic toxicity, in the setting of vascular stenosis. This concept has already been successfully validated through the recent TAXUS clinical trials which demonstrated that paclitaxel-coated coronary stents can result in a significant reduction in in-stent restenosis as compared with bare metal stents [9]. An alternative mode of local delivery of therapeutic agents to the vascular wall is through the perivasular approach. An \textit{ex vivo} model has demonstrated that paclitaxel applied to the outside of the arterial vessel wall diffuses through the entire arterial wall thickness, with paclitaxel being present within the media and intima in addition to the adventitia [10]. In addition, such a perivascular drug delivery approach has been successfully used to attenuate post-angioplasty stenosis using agents such as nitric oxide, calcium antagonists, dexamethasone and tyrphostins [11–15].

We have previously described in detail [16,17] the preparation, physicochemical properties and \textit{in vitro} release profile of paclitaxel from ethylene vinyl acetate (EVA) polymeric matrices (wraps) containing 5% w/w paclitaxel and 15% w/w polyethylene glycol (PEG)4000 as a channelling agent (the latter alters the release profile of paclitaxel from the EVA). This particular concentration of PEG4000 allows the paclitaxel release profile (burst release (40% of loaded paclitaxel) in the first 72 h followed by a slower continuous release profile (10% of loaded paclitaxel) over the next 11 days) (Figure 1), to mirror the profile of cellular activation in polytetrafluoroethylene (PTFE) graft stenosis. In addition, we have demonstrated a significant inhibition of all the three cell types present in the lesion of VNH (smooth muscle cells, fibroblasts and microvessel endothelial cells) \textit{in vitro}, when exposed to these paclitaxel-loaded polymers [16,17].

Following on from these experiments, the aim of our current study was to describe the \textit{in vivo} safety and efficacy profile of these paclitaxel-loaded wraps in a validated pig model of arteriovenous graft stenosis [18]. Demonstration of efficacy in this pig model, would allow us to move forward in developing this intervention for clinical use.

\textbf{Subjects and methods}

\textit{Development of a local polymeric delivery system for paclitaxel}

Polymeric films containing 5% w/w paclitaxel (Sigma Chemical Co., St Louis, MO, USA), 15% PEG4000; (Dow Chemical Co., Danbury, CT, USA) and 80% EVA (Elvax 40, Du Pont Co., Wilmington, DE, USA) were prepared by dissolution in methylene chloride followed by a solvent casting technique. Bubble-free EVA/paclitaxel/PEG solutions were poured into PTFE evaporation dishes, air-dried and cast into polymeric matrices. These matrices were then cut into rectangular pieces (1.5 × 0.6 cm, 0.3 mm thickness) for the \textit{in vivo} animal experiments. Furthermore, in order to provide unidirectional release of paclitaxel in the setting of perivascular wraps (into the graft and vein and not into the surrounding tissues), one side of the polymer was coated with methylene chloride. We have previously demonstrated that this particular concentration of EVA, PEG4000 (as a channelling agent) and paclitaxel results in an initial burst release followed by a slower long-term release profile that could be well-suited to the pathobiology of VNH in the setting of haemodialysis vascular access dysfunction (Figure 1) [16,17].

\textit{In vivo experiments with paclitaxel-loaded wraps in a swine model of arteriovenous graft stenosis}

\textit{Placement of arteriovenous grafts.} 6.5 cm long, 4 mm internal diameter PTFE grafts were placed between the femoral artery and vein in nine pigs as previously described.
in our validated animal model [18]. Heparin (200 µ/kg i.v.) was administered during surgery. All animals received aspirin (325 mg) from day –1 until the day of graft harvest.

**Perivascular paclitaxel-loaded wraps.** Following placement of the arteriovenous grafts, a prepared sterile paclitaxel-loaded wrap (5% paclitaxel + 15% PEG4000 + 80% EVA) was carefully placed around the graft-vein anastomosis on one side (Figure 2). A prepared sterile control wrap (15% PEG4000 + 85% EVA) was placed around the contralateral graft-vein anastomosis. The control and paclitaxel wraps were placed in either the right or left groin in a random fashion. The wraps were weighed prior to insertion in order to ensure uniform administration of paclitaxel dose. Special care was taken to secure the wraps at the graft-vein anastomosis in a configuration that allowed expansion of the blood-filled vein without constriction, but also kept the non-coated side of the polymer in direct contact with the graft-vein anastomosis.

**Assessment of toxicity.** All animals were examined daily for evidence of graft patency, wound inflammation or anastomotic breakdown. Blood samples were tested at baseline, 30 min, 1 h, 2 days and between 10 and 38 days (depending on the time of assigned sacrifice) for evidence of systemic paclitaxel in six pigs. Blood samples were also tested in four pigs at baseline (time of receipt into husbandry), on the day of surgery and 3 days post-surgery for white blood cell, platelet and haemoglobin levels, in order to assess for any haematological toxicity from the perivascular paclitaxel wraps. A macroscopic examination for evidence of tissue necrosis was performed at the time of sacrifice followed by an evaluation of haematoxylin and eosin (H and E) sections for microscopic evidence of local tissue necrosis.

**Sacrifice of animals.** Animals were sacrificed at three different time points, early, middle and late. The ‘early’ time point animals (n = 4) were sacrificed between days 10 and 11 (10, 10, 10, 11, respectively); the ‘middle’ time point animals (n = 2) were sacrificed on days 23 and 24 (23, 24, respectively) and the ‘late’ time point animals (n = 3) between days 32 and 38 (32, 37, 38, respectively). At the time of sacrifice, the grafts, together with the attached artery and vein were carefully dissected out and fixed in formalin. The graft-vein anastomosis was then cut into 3 mm blocks (Figure 3A) which were embedded in paraffin and cut into 3 micron sections.

**Histomorphometric analyses.** H and E sections of 3 micron thick were examined using standard histomorphometric techniques. Venous stenosis at the graft-vein anastomosis (Figure 3A) was assessed as described previously by our group [18]. Briefly, measurements were made of the neointimal hyperplasia (NH) area (black area in Figure 3B) and of the entire luminal area (black + white area in Figure 3B) subtended by PTFE graft (a line was drawn between the two ends of the graft; see AB in Figure 3B). Percentage luminal stenosis was calculated using the formula (black/white + black) x 100. Percentage luminal stenosis at the graft-vein anastomosis was calculated for each pig as a mean of all adequate sections. An adequate section for histomorphometric analysis was defined as a section which included a semicircle of graft and a semicircle of attached vein. This was by definition, a section from one of the blocks cut at the level of the vertical lines AB in Figure 3A.

**Statistics**

The mean percentage luminal stenosis for the control wraps was compared with the mean percentage luminal stenosis for the paclitaxel wraps using a paired t-test. These analyses were performed for all the three time periods (early, middle and late) combined, and also for just the middle and late time periods together (since there was no luminal stenosis in the control animals at the early time point). We also analysed the data for differences in luminal stenosis between the control and paclitaxel-treated animals using a Mann–Whitney non-parametric test. A P-value of <0.05 was considered to be significant.

![Fig. 2. Placement of perivascular paclitaxel wraps: Demonstration of a perivascular paclitaxel-loaded wrap in the appropriate position at the graft-vein anastomosis in our pig model of arteriovenous graft stenosis.](https://example.com/fig2)
Results

Local and systemic toxicity

There was no evidence of significant clinical, macroscopic (visual examination at the time of sacrifice) or microscopic (Figure 4A–D) local toxicity from the perivascular paclitaxel wraps. Notably, the tissue surrounding the paclitaxel-eluting wraps was in most cases (but not always) far more hypocellular or acellular as compared with the tissue in the region of the control wraps. The predominantly fibrinous material that layered the paclitaxel-eluting wraps did not appear to predispose to haematoma formation.

Of the 17 pigs that we have experimented with to date (including those that were used in an initial feasibility study on the use of wraps and not included in this article), overall we have had one anastomotic breakdown only. This occurred in a paclitaxel-treated pig. Paclitaxel was not detected in the systemic circulation of the six pigs tested for paclitaxel levels after placement of the wraps. In particular, there was no evidence of systemic paclitaxel at day 2 when near maximal systemic concentrations of paclitaxel would have been expected based on the pharmacokinetic release profile of paclitaxel in our local delivery system (Figure 1). There was also no evidence of any haematological toxicity (results not shown) in the four pigs that had complete blood counts done as described in the ‘Subjects and methods’ section.

Percentage luminal stenosis

Percentage luminal stenosis in the paclitaxel-treated animals as compared with the control animals (at the early, middle and late time points) is summarized in Figure 5. Note that there was no luminal stenosis (<0.5%) at any of the graft-vein anastomoses treated with the paclitaxel-loaded wraps (85% EVA + 5% paclitaxel + 15% PEG4000). The mean luminal stenosis in the control animals varied between 1.34 ± 1.07% in the early time point group (too early for stenosis) to 20.59 ± 8.89% in the middle time point group and 49.46 ± 28.87% in the late time point group. Although clinically significant, these differences did not reach statistical significance due to the small number of animals in each sub-group and also as a result of the expected biological variation in the control group. When a combined analysis was performed on animals in the middle and late time point group (time points at which clinically relevant luminal stenosis was expected in the control animals) there was a trend towards a statistically significant result (37.90 ± 17.55% luminal stenosis in the controls vs 0.10 ± 0.10% in the paclitaxel group; \( P = 0.096 \)). The use of a non-parametric Mann–Whitney test, however, was able to demonstrate a statistically significant difference between the control and paclitaxel-treated animals (\( P = 0.0341 \)). Figure 6A and B show some representative examples of inhibition of VNH at the graft-vein anastomosis with the perivascular paclitaxel and control wraps.

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**Fig. 4.** Microscopy for assessment of local tissue necrosis on the external graft surface with the paclitaxel-eluting wraps: A and B demonstrate a lack of toxicity at an ‘early’ time point (10 days) following the burst phase in a paclitaxel-treated animal (B). C and D document a long-term lack of necrosis in a paclitaxel-treated animal at the ‘late’ time point (37 days, D). Note that the paclitaxel-eluting wraps in both cases (B and D) appear to be covered with a hypocellular/acellular fibrinous material (asterisks) as opposed to a more cellular tissue in the controls (arrows). Also note the presence of a mononuclear cell layer in the control animals (A, arrowheads) as compared with C and D (paclitaxel-eluting wraps).
Discussion

In the above paragraphs, we have documented that paclitaxel-loaded perivascular wraps can completely block VNH in an in vivo pig model of arteriovenous graft stenosis, that is very similar to the lesion of venous stenosis in haemodialysis patients [18]. We believe that these are important results as they provide the proof of principle for the use of perivascular drug delivery systems in the setting of haemodialysis vascular access dysfunction, and open the way for the clinical development of local perivascular paclitaxel drug delivery systems, both in the specific setting of arteriovenous dialysis graft stenosis and in other clinical models of PTFE graft stenosis.

Some key aspects of the above work are discussed below:

Advantages of perivascular drug delivery systems for vascular stenosis

The traditional pathogenetic view of vascular stenosis has always been that there is a migration of smooth muscle cells from the media into the intima, where they proliferate to form the lesion of neointimal hyperplasia (NH) [19]. Recent studies, however, have challenged this paradigm and emphasized the importance of the adventitia in final luminal stenosis. Thus, Shi and others [20–22] have demonstrated that following experimental coronary angioplasty or saphenous vein bypass grafting, there is a migration of adventitial fibroblasts from the adventitia, through the media and into the intima. Once in the intima, these cells change their phenotype to that of myofibroblasts (through the acquisition of α-smooth muscle actin expression) and contribute to the total neointimal volume [20]. Our own data in the setting of PTFE dialysis access grafts in haemodialysis patients also support this paradigm [23] as does a recent study in an arteriovenous graft model by Misra et al. [24].

Other authors have demonstrated that final luminal stenosis is dependent not only on the amount of NH, but also on the pattern of vascular expansion or constriction [25]. Thus, a combination of aggressive NH and vascular constriction, would result in maximal luminal stenosis; on the other hand, aggressive NH in combination with vascular expansion would result in minimal luminal stenosis. Although, the factors responsible for vascular constriction resulting in adverse remodelling are not clearly defined [26], with the exception of some data on the role of transforming growth factor-β [27], it has been demonstrated that both experimental coronary angioplasty and saphenous vein graft surgery result in an inflammatory response (myofibroblasts and angiogenesis) within the adventitia which could then result in vascular constriction [28–30]. Perivascular drug delivery with the appropriate therapeutic agents could be the ideal way to modulate adventitial inflammation and constriction.

Thus, we speculate that an ‘outside-in’ approach with a drug eluting perivascular polymer could be the ideal mode of local delivery for this specific pathological process. In particular, we felt that (i) vascular remodelling which may be dependent on adventitial angiogenesis and constriction, and (ii) the migration of myofibroblasts or fibroblasts from the adventitia to the intima are best targeted through the use of perivascular paclitaxel wraps. Such an outside-in...
analyses in a pig model, using a different polymer.

Clinicopathological correlations

By analysing data at the ‘early (10 days), middle (24 days) and late (36 days)’ time points, our results allow us to glean important information about this drug delivery system in the context of the natural biology of venous stenosis in our pig model [18]. Thus, the lack of both macroscopic and microscopic evidence of tissue necrosis and subsequent inflammation at the early time points (10 days; Figure 4), confirms that the initial burst release (72 h) of paclitaxel from the perivascular polymers (Figure 1) does not have a detrimental downstream effect. The complete absence of VNH at the middle time point (24 days) as compared with a 20.58% stenosis in the control grafts, is proof of the efficacy of a combination of the paclitaxel burst effect followed by continuous gradual release. The most important result in our minds, however, is the complete absence of VNH at the late time point (36 days). This last result suggests that the continuous release of relatively small amounts of paclitaxel (well after the initial burst release is over) is sufficient to prevent neointimal hyperplasia (note that small amounts of paclitaxel released at the graft-vein anastomosis could easily achieve local concentrations that are sufficient to block cellular proliferation and migration).

In addition, extrapolation of the release profile of paclitaxel based on the amount of paclitaxel initially added to the polymer, suggests that small amounts of paclitaxel will continue to be released locally for ~3 months. This prolonged release of paclitaxel (as compared with the 30 days release profile for the paclitaxel eluting coronary stents) is particularly important in the context of haemodialysis vascular access dysfunction, where there is continuous ongoing haemodynamic stress at the graft-vein anastomosis as opposed to a single episode of endothelial and smooth muscle injury in the setting of coronary angioplasty. In particular, we believe that 3 months of paclitaxel release could be long enough to have a significant clinical impact in the setting of PTFE dialysis access grafts, where the current 1 year primary patency is a dismal 50% at 1 year.

Our enthusiasm for the use of paclitaxel in a local perivascular delivery system for venous stenosis is supported by a recent successful study (five dogs) on the feasibility of using a percutaneously injected perivascular, paclitaxel-containing gel, to inhibit VNH in a canine model of arteriovenous graft stenosis [32]. We believe that our current study, both complements and adds to these earlier results. In contrast to the study by Masaki et al. [32] which used a semiquantitative analysis in a canine model of arteriovenous graft stenosis, we have performed histomorphometric analyses in a pig model, using a different polymer (EVA rather than ReGel). The key message from both our studies, therefore, appears to be that perivascular paclitaxel-releasing polymers and wraps can effectively block VNH and vascular stenosis, regardless of differences in the animal model or the polymer delivery system used. We believe that this dual confirmation (using different model systems and delivery techniques) is critically important, as we move towards the clinical application of these novel therapeutic concepts in the setting of haemodialysis vascular access dysfunction.

Finally, but perhaps most importantly, we strongly believe that clinical haemodialysis vascular access grafts and fistulae could be the ideal clinical model for our paclitaxel-loaded perivascular wraps, because of the following reasons:

(i) haemodialysis access grafts and fistulae are superficially located and the wraps can therefore be easily placed at the time of surgery.

(ii) haemodialysis access grafts have an aggressive natural history of vascular stenosis which could mean that clinical trials of novel therapies could be rapidly conducted in far smaller numbers of patients and over a shorter follow-up period as compared with the large post-coronary angioplasty studies.

In conclusion, we have demonstrated a complete absence of luminal stenosis at the graft-vein anastomosis, in PTFE arteriovenous grafts treated with perivascular paclitaxel-loaded wraps. We believe that the rapid translation of this technology to the clinical setting of haemodialysis vascular access dysfunction could significantly reduce both the clinical morbidity and the huge financial burden associated with haemodialysis vascular access dysfunction. In addition, we hope that this novel paradigm for the treatment and prevention of vascular stenosis can also be successfully applied to other clinical models of vascular stenosis.

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Conflict of interest statement. None declared.

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