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High concentration of paclitaxel coated graft for hemodialysis elicits a severe foreign body response in a porcine model

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Abstract

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The most crucial reason for vascular access dysfunction in hemodialysis patients is stenosis caused by neointimal hyperplasia. Until now, paclitaxel has been shown to act as a great anti-proliferative agent and effectively lowers stenosis on graft-venous anastomosis sites. Dipping method allows us to obtain paclitaxel coated Arteriovenous (AV) expanded polytetrafluoroethylene (ePTFE) grafts in a quick, uniform and facile manner. As the whole graft surface elutes paclitaxel upon implantation by systemic circulation, I ought to validate how various amounts of paclitaxel release affect a porcine system. I coated the following concentrations of

paclitaxel for study- Control (0 ug/mm^2), Group 1 (0.14ug/mm^2), Group 2 (0.28ug/mm^2), Group 3 (0.51ug/mm^2), Group4 (1.11ug/mm^2), and Group 5 (2.22ug/mm^2). This work uncovers that in general, paclitaxel coated grafts revealed high level of patency compared to the control group in which grafts were almost all occluded, however; at high concentrations of paclitaxel, the surrounding tissue and the graft retained little affinity. I attribute this for a foreign body response and hence arise necessity to tune the amount of paclitaxel in order to minimize both stenosis and foreign body response.

Keywords: *Paclitaxel, AV graft, neointimal hyperplasia, foreign body response, patency, hemodialysis*

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1. Introduction

1.1.1 Different types of vascular access

Patients with chronic renal disease requiring hemodialysis therapy need a stable vascular access in order to withdraw blood at a rate of approximately 300mL/min from the patient. This rate is higher than and lower than the rate of blood flow in vein and artery respectively. In most cases, patients requisite a permanent vascular access rather than a temporary means, thus it is necessary to connect the artery and the vein for the efficient hemodialysis. Types of the permanent access for a long-term dialysis are mainly arterio-venous (AV) fistulas, and AV grafts. Different outcomes, degrees of patency, and types of complications arise from each type of the vascular access [1, 2].

1.1.2 Arteriovenous Fistula

AV fistula can be categorized into two subgroups based on the use of materials. The autogenous AV fistula is constructed only with native material and the non-autogenous AV fistula is utilized alternatively when there are insufficient or unavailable veins to build a vascular access. The non-autogenous AV fistula is also known as AV graft as surgeons rely on graft materials. If possible, AV graft implantation is avoided due to relatively lower outcomes [2, 3]. Fistula can be built over the entire body, however; the upper extremity is preferred due to lower complication.

Surgeons prefer to select the vessels at the most distal site available in the case of failing the first access and to preserve available sites for the future inquiry [1]. Compared to the AV graft, AV fistulas exhibit higher patency although patency rates may differ among different research teams due to human factors involving surgical skills and patient pool categories [3].

1.1.3 Arteriovenous Graft

For most patients, AV fistula is an ideal method and it directly connects aforementioned vessels. It rarely induces inflammation and provides guaranteed safety as no foreign material is introduced [2]. On the other hand, AV graft is utilized for the patients of whom the vessel is weakened by aging or a disease such as diabetes. Weak vessels are unable to withstand numerous needle punctures from a hemodialysis machine and thereby a graft is inserted in between the vein and the artery. The inlet and the outlet of the hemodialysis machine are then connected through the graft so it prevents the vessels to be worn. Therefore AV graft is preferred over fistula in the cases of such inquiry, however; it has been reported that AV graft can be more prone to infections and/or stenosis [4-6]. Synthetic materials such as expanded polytetrafluoroethylene (ePTFE), silicon, or biological materials like homologous veins, bovine or sheep vessels may be used as grafts based on that as each material provides its own pros and cons [1]. EPTFE is the most widely used material. In the USA, nearly 83% of the hemodialysis patients are using PTFE grafts [5]. Grafts can be implanted either in the forearm or in

the upper arm in a straight or with a loop configuration. If the graft is placed in a loop, cautious observation is needed to avoid graft twisting or kinking. When complications take place, it is almost impossible to rescue graft and the graft should be eliminated surgically. Although failure of AV fistula in prior is irrelevant with the following graft failure, previous graft failure increases the risk of following graft failure substantially [2, 6]. In this study, AV graft is used.

1.2.1 Complications

Hemodynamic complications that may be arising from AV fistula and AV graft are stenosis and thrombosis, aneurysm formation, steal syndrome, ischemia, and heart failure. Non-hemodynamic complications include inflammations [2, 7]. This study mainly deals with stenosis and inflammation complications. Effects of thrombosis is excluded using heparin solution which eliminates blood clots. For further information on symptoms unstated in detail, refer to: Radojica Stolic (2013) *Med Princ* Pract: **22**.

1.2.2 Stenosis

Stenosis is a pathological condition of blood vessel narrowing. In AV fistula or graft, stenosis mostly occur in the anastomosis site and in the draining vein [8]. This phenomenon can be reasoned by changes in of the flow property in the vein before and after the vascular access surgery. In the natural vein, blood pressure is relatively low at about 20 mmHg and blood flow is not pulsatile (pulse-variable), and wall shear stress is below 0.76 Pa. However;

upon vascular access creation, pressure mushrooms from 60 to 120 mmHg, flow is pulsatile, and wall shear stress becomes above 0.76 Pa[1, 7]. Neointimal hyperplasia is the underlying mechanism of stenosis. In order to understand the process of neointimal hyperplasia, the vein structure should be studied. The most inner layer is called the intima and it is consisted of a single layer of endothelial cells, the basement membrane, followed by the sub-endothelial matrix. In the sub-endothelial matrix, glycoproteins and connective tissue elements are in the upper layer. Tunica media is below the aforementioned layer and in tunica media, vascular smooth muscle cells (VSMC) reside. Adventitia is the thick outermost layer consisted of longitudinally oriented collagens and scattered fibroblasts [1, 2]. During the formation of neointimal hyperplasia, VSMC in the tunica media migrate into the intima layer and it is considered neointimal hyperplasia is present when 20% of VSMC migrated into the intimal region, proliferated, and excreted extracellular matrix comprising 60-80% of the intima. Neointimal hyperplasia can be triggered by various aspects. It is initiated from injuries such as incisions from suturing. Factors like abnormal wall shear stress, high venous pressure, cyclic stretch, mismatch of elastic property between the vessel and the graft, and resultant angle between the vein and the graft can also elicit intima formation at various degrees [1-3, 7, 9]. Stenosis formation may lead to thrombosis and it rises importance to catch it in the earlier stage. There is a higher risk of causing thrombosis as stenosis formation increases [1]. Doppler ultrasound is a non-invasive tool to measure a degree of stenosis.

Peak systolic velocity in the Doppler spectrum is regarded as a fairly accurate parameter for detecting stenosis [10]. If a degree of stenosis formation is severe, it should be treated to circumvent thrombosis occurrence. In order to remove stenosis, an inflatable balloon angioplasty is inserted to dilate the narrowed region. If the stenosis was removed inadequately, a stent is placed over an inflatable balloon and upon inflating, stent is widened and fitted to the diameter of the vessel. Nevertheless, restenosis occurrence is quite frequent spanning from 15 to 40%. If angioplasty treatment is insufficient, surgical removal is required [1-3, 10].

1.3.1 Factors developing Neointimal Hyperplasia

Potentially there are several factors that may induce neointimal hyperplasia development such as increased turbulent flow, deficiency of endothelial cells, compliance mismatch between the native vessel and the graft, vessel damage, endothelial injury caused by high shear force, and growth factor release due to mechanical injury [1-3].

1.3.2 Surgical Interventions

As mentioned before, neointimal hyperplasia is developed most frequently at the anastomosis site and/or in the draining vein, especially along the suture line. Surgical trauma and the endothelial injury unquestionably initiated intimal hyperplasia [3,10].

1.4.1 Anti-proliferative agent coating

Anti-proliferative agent coating has been actively studied to reduce stenosis formation without surgical treatment. Its primary mechanism is to inhibit or to retard proliferation of the VSMC in the intima region. There are two types of anti-proliferative agent: rapamycin and paclitaxel. Rapamycin is known to inhibit interleukin-2 induced phosphorylation and activation of target kinases [11]. In the case of paclitaxel, its main mechanism is to stabilize microtubules in the cytosol during the normal cell division. Both rapamycin and paclitaxel are regarded as the most effective agents aiming to inhibit stenosis by anti-proliferative roles. For such reason, they have been widely used for graft coating or other biomaterials such as stents in order to inhibit stenosis formation [12]. However, previous studies, the randomized trial data, revealed that paclitaxel coated material is more effective than rapamycin coated one, and thence paclitaxel is selected for coating AV graft in this study [13]. Up to date, many researches have proved that paclitaxel coated graft greatly retards neointimal tissue growth on the implantation site and remains the graft interior highly patent [14, 15].

1.4.2 Foreign body response

Previous studies have reported that paclitaxel localization on the outer surface of the graft may involve unwanted effects such as infections, seromas, pseudoaneurysms, or hematomas because of its toxic property that prevents cellular proliferation of myofibroblasts on the graft surface which

can develop an empty space between the surrounding tissue and the graft [16, 17]. Following the implantation, the interface between the material (e.g. graft) and the surrounding tissue is created. Upon material implantation and the associated injuries, inflammatory cascade and wound healing process are triggered. An inflammatory cascade is dependent on the exposure time of the material and it is a typical type of foreign body response. An acute phase and a chronic phase comprise the inflammatory cascade [18]. During the acute phase lasting from hours to days, provisional matrix is formed and cleaning of the injury site by neutrophils takes place. Leukocytes adhere to the wall of a blood vessel to infiltrate and numerous tissue and blood proteins, such as growth factors and cytokines, are released. If the injury is not recovered and inflammatory phase is persistent, the chronic phase is followed gathering monocytes which differentiate into macrophages and lymphocytes. Blood vessels proliferate and connective tissue is reconstructed in the affected area to form granulation which then is replace by extracellular matrix in a normal wound healing process. However, the implanted material is too big for phagocytosis and frustrated macrophages fuse together to form a multinucleated giant cell around the material. In the end stage of the foreign body response, fibroblasts are called and blood vessels form to produce collagenous fibrous tissue and it completely walls of the material. As a result, interactions between the surrounding tissue and the material are prevented and the fibrous capsule containing the material, water, other protein components, and wastes is called a cyst [18-20].

1.5 Coating methods and previous studies

Previous works have shown multiple ways coating AV grafts. Firstly, grafts were coated by dipping method. Dipping method is immersing a graft in a paclitaxel solution to coat. It is a cost-effective and facile way however; outer surface is also coated with paclitaxel which prevents cellular adhesion onto the surface. As continuous exposure of the surface of the graft due to paclitaxel may trigger the inflammatory cascade so paclitaxel-PLGA loading for sustained release was applied, however, animal experiments were not performed [21, 22]. As stenosis formation at the anastomosis site had occurred within 3 cm, terminal coating method was followed [23]. Tough it effectively reduced stenosis formation, still there was a chance of inflammation and thus, coating the inner surface of the graft to minimize poor prognosis was employed [16]. Inner coating requires its own coating device which was manufactured manually. As paclitaxel solution is pumped up by a syringe pump pulsatily and a nozzle is moving for coating, uneven paclitaxel distribution was frequently observed. It has been proven that inner coating method exhibits higher affinity with the surrounding tissue and minimizes stenosis at the same time, however; more optimization is required to obtain even coating. Hence in this study, primary coating method is used to investigate the problematic inflammation degrees with different concentrations of paclitaxel which renders a good starting point to connect the relationship between paclitaxel eluting material and a cyst formation.

1.6 Research objective and clinical relevance

So far, there is no information that deal with both level of patency and adverse side effects that may be accompanied by depending on different concentrations of paclitaxel. In addition, aside from grafts, there are numerous kinds of biomaterials eluting anti-proliferative agents such as paclitaxel or rapamycin. In previous studies, cyst formations were observed possibly due to adverse inflammatory responses in a porcine model. Also there were incidences of failure in clinical trial due to heavy inflammations while using an anti-proliferative drug eluting material. This study holds its clinical relevance in such a way that a close relationship is built between the level of stenosis and possible inflammatory responses. In this study, I utilize a dip coating method to coat whole ePTFE grafts with various concentrations of paclitaxel and investigate how they induce different fates spanning from stenosis reduction and/ or inflammation in a porcine model, and which one can minimize related pathology [24].

2. Methods

2.1 Materials

Paclitaxel (Genexol®) was purchased from Samyang Genex Inc, South Korea. The ePTFE vascular grafts (IMPRA, F4006C) were purchased from Bard Peripheral Vascular Inc, USA. High performance liquid chromatography (HPLC)-grade acetone and acetonitrile were obtained from Fisher Scientific, USA. Tween 20 was purchased from Sigma Cheminal Co, and phosphate-buffered saline (PBS) was obtained from Cambrex Corporation, USA.

2.2 Preparation of paclitaxel-coated ePTFE grafts

Paclitaxel-coated vascular grafts were made using a single dipping method. Briefly, paclitaxel was dissolved in acetone to a concentration of 0.25, 0.5, 1.0, 2.0 or 4.0 mg/mL in polypropylene tubes, respectively. Each of the ePTFE vascular grafts, 6 mm in diameter and 20 cm long, was dipped into one of these solutions and incubated for 30 min at 37℃ in a roller incubator (Combi-H; FinePCR, Korea). The paclitaxel-coated ePTFE grafts were then air-dried and kept under vacuum overnight to remove the solvent completely. These grafts were sterilized in ethylene oxide gas before use. The corresponding amounts of paclitaxel on the grafts for the coating solution concentrations of 0.25, 0.5, 1.0, 2.0 or 4.0 mg/mL were 0.14, 0.28, 0.51, 1.11 and 2.22 ug/mm^2, respectively.

2.3 In vitro release test

For the in vitro release test, PBS (pH 7.4) containing 0.05% (w/v) Tween-20 was used as the drug release medium. Paclitaxel-coated vascular grafts with a length of 5 cm were soaked in polypropylene tubes with 8 mL of release medium and shaken at 37℃ and 20 rpm in a roller incubator. At designated time points over a period of 28 days, release medium was removed completely from the tubes and analyzed by HPLC (Agilent 1200 Series, USA) using a 4.6 x 150 mm C18 reverse-phase column and a UV detector set at 227 nm. HPLC analysis was performed using a mobile phase of water: acetonitrile (50:50 v/v) at a flow rate of 0.8 mL/min. Under these conditions, the peak of paclitaxel was eluted at 9.5 minutes.

2.4 Experimental animals and operative technique

25 female Landrace pigs, weighing 50 ± 7 kg, received one of six types of grafts between the common carotid artery and the external jugular vein: grafts without paclitaxel coating (control, n = 6); grafts with paclitaxel coating at a dose density of 0.14 μg/mm^2 (group 1, n = 4), 0.28 μg/mm^2 (group 2, n = 4), 0.51 μg/mm^2 (group 3, n = 4), 1.11 μg/mm^2 (group 4, n = 4) and 2.22 μg/mm^2 (group 5, n = 3). The animals were maintained in standard animal care facilities at Samsung Biomedical Research Institute (SBRI). This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of SBRI. Each operating procedure

conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

Pigs were anesthetized with intramuscular ketamine HCl (20 mg/kg) and xylazine HCl (2 mg/kg). Then, they were incubated and ventilated with a mixture of O_2 and air (1:2) containing isoflurane (2%) for maintenance anesthesia. Vecuronium bromide (0.1 mg/kg) was administered continuously through an ear vein.

I adopted the animal model proposed by Rotmans et al. [25] Following standard surgical cleansing, a longitudinal incision was made in the lateral side of the neck along the sternocleidomastoid muscle. The common carotid artery and the external jugular vein were exposed and then heparin was given intravenously at 100 IU/kg before vessel manipulation. The common carotid artery was clamped using vessel loops and an 8-mm arteriotomy was made. An end-to-side anastomosis was made at approximately 45° using a 6-0 polypropylene suture. Venous anastomoses were created in a similar manner. When the animals were euthanized at 6 weeks after operation, all the explantation process was done in anesthetized state. The implanted grafts were excised along with adjacent vessels, and immediately placed in heparin-containing PBS (10,000 IU/L), in order to remove the remaining blood from the vessel. From surgery day to the day of euthanasia, all pigs were administered aspirin (100 mg/day) and clopidogrel (PLAVIX®, Sanofi Aventis, France) 75 mg/day.

2.5 Paclitaxel amount in explanted graft

In order to determine the amount of paclitaxel remaining in the graft implanted in a porcine for a 6-week-observation, the explanted graft was washed twice with normal saline and incubated in HPLC-grade methanol for 2 hours. The solution was then centrifuged down for 20 min and the supernatant was used for the analysis.

2.6 Tissue preparation and morphometry

The excised specimens were immersion fixed in 10% neutral-buffered formalin (NBF) for at least 24 h and embedded in paraffin. Sections (5 μm) of veins 1-cm proximal and distal to anastomosis were prepared perpendicular to the blood flow. Serial sections were taken to obtain cross sections around the center of the graft–venous anastomosis, and three sections were analyzed in order to determine the percentage of luminal stenosis, as shown in Figure 1.

All sections were stained with Masson Trichrome. The areas of intima and media could be manually traced on captured images obtained with an Aperio ImageScope (Aperio, USA). To compare stenosis among the control and paclitaxel-coated groups, the percentage of luminal stenosis was calculated from the area of neointima divided by the total luminal area inside grafts and vascular tissues. I measured the percentages of luminal stenoses in three different sections from each graft-venous anastomosis site, and the mean

values for these three sections were analyzed to compare between the groups.

2.7 Immunohistochemical staining

Sections (5 μm) of paraffin-embedded tissues were immunostained with antibodies to alpha-smooth muscle actin (SMA; Abcam PLC, UK), vimentin (AbD Serotec, UK) and desmin (GeneTex, USA) using Vectastain ABC kits (Vector Laboratories, USA) according to the manufacturer's instructions. Briefly, sections were deparaffinized and hydrated through a xylene and graded alcohol series. After antigen retrieval in antigen unmasking solution (Vector Laboratories) for 15 min, the slides were incubated in 0.3% H_2O_2 for 30 min, in normal blocking serum for 20 min, in primary antibody overnight at 4 °C, in biotinylated secondary antibody for 30 min, in Vectastain ABC reagent for 30 min and in peroxidase substrate solution. The sections were then counterstained with hematoxylin, dehydrated through a graded alcohol series. To analyze cellular phenotypes for adjacent tissue outside the graft wall, semiquantitative scoring of all sections was performed on a scale from 0 to 4+, which corresponded to the percentage of positive cells for the specific marker in several different areas (0 = 0-10% positive; 1+ = 11-25% positive; 2+ = 26-50% positive; 3+ = 51-75% positive; and 4+ = 76-100% positive) [26].

2.8 Statistics

All data are presented as mean values ± the standard errors. Kruskal-Wallis tests were used to compare the percentages of luminal stenosis among groups; $P<0.05$ was considered statistically significant. I used SPSS 18.0 software (SPSS Inc., USA) for all statistical analyses.

3. RESULTS

3.1.1 In vitro paclitaxel release profiles

The in vitro drug release profiles for 28 days were measured to compare release patterns according to the coated amount of paclitaxel on the ePTFE grafts, as shown in Figure 2. A burst release of paclitaxel was followed by a slower sustained release, but less than 10% of the initial amount of the loaded drug was released after 28 days. Interestingly, in vitro release amount of paclitaxel tend to decrease significantly after sterilization with ethylene oxide gas, as in these case.

3.1.2 In vivo paclitaxel release profiles

During a 6-week-incubation, paclitaxel coated on the graft in group 1 and group 5 was all released by 99 % and 98 % respectively.

3.2.1 Animal Experiments and histomorphometry

I measured the luminal narrowing at the graft-venous anastomosis site.

3.2.2 Percentage luminal stenosis

As shown in Figure 3, pale blue regions were hyperplastic tissues in the inner vascular graft. The graft appeared to be a thick layer of red. Although lower venous region of cross-section was patent, the upper graft region might be entirely occluded. If the percentage of luminal stenosis at the graft-venous anastomosis site was more than 50%, the blood flow was significantly reduced, and the middle region of implanted graft was often occluded much more. In this case, the blood flow could not be measured

using a Doppler sonograph. Therefore, I considered that the implanted graft was occluded when the percentage of luminal stenosis was more than 50%. Depending on this assumption, five of the six control grafts were thought to be occluded even though the percentages of luminal stenosis were not 100%. Statistical values on % stenosis are listed in Table 1. If the luminal stenosis area occupies less than 50% of the total graft area in the cross section, the graft is regarded patent. Average % stenosis of the control group indicates few patency was observed while most of the grafts coated with paclitaxel exhibited ~100% patency. % Stenosis in group 5 was found to be the least whereas average % stenosis of group 1 to 4 lied within a moderate range from 14.5 - 21.3%. It should be noted that not all P^a values were less than 0.05 due to small n numbers (n= 3-6) in comparison with control, thus I considered % stenosis of group 1 to 4 to be of similarity. As shown in Figure 4, percentage of luminal stenosis drastically dropped from the control group to the ones coated with paclitaxel. Dash line indicated with * illustrates the mean values and only group 2 and 5 are statistically significant with unpaired Student's *t* test.

3.2.3 Thrombosis

In 25 pigs including the control group, there was no case that was occluded due to thrombosis in this animal study. Therefore, I needed not to consider the influence of thrombosis to evaluate the percentage of luminal stenosis. To remove the thrombus which may result from surgical procedure, the

excised ePTFE grafts and adjacent vessels were immediately placed in heparin-containing PBS (10,000 IU/L) to washout the remaining blood.

3.2.4 Immunohistochemistry

Immunohistochemistry for differentiating cellular types of the tissue surrounding the graft was performed. Three types of antibodies were employed to detect the type of cells as indicated in Table 2; *a*-SMA for detection of smooth muscle cells and myofibroblasts, vimentin for myofibroblasts and fibroblasts, and lastly desmin for smooth muscle cells only. Control group in the first row reveals high number of myofibroblasts and fibroblasts but relatively less amount of smooth muscle cells. In general, control group achieved successful tissue adhesion on to the graft. Group 2 (0.51 ug/mm^2) exhibits better tissue adhesion than Group 1 (0.14 ug/mm^2) based on the number of cells stained. Group 3 (2.22 ug/mm^2) scarcely retained any type of cells and poor tissue affinity on the graft was observed. It should be noted that the tissue affinity does not always decrease as the concentration of paclitaxel increases, which rises importance on adjusting paclitaxel amount.

4. DISCUSSION

Luminal neointimal tissue formation after implanting a graft is associated with multiple interactions among cellular components and non-cellular components such as vessel walls and blood [27]. Such that, upon implantation, inflammatory reactions take place and excessive blood flow into the scarred region that a great number of migratory cells gather at the graft-venous site (Figure 1) [25, 27, 28]. It has been widely studied with biomaterials coated with paclitaxel, e.g. stents or grafts as paclitaxel effectively enhances luminal patency and minimizes occlusion even at a low concentration [16, 29]. In this work, effects of various doses of paclitaxel coated grafts on stenosis formation were compared and I found some interesting results [30, 31]. I observed that paclitaxel coated grafts notably suppressed neointimal tissue formation and increased patency compared to the control group. There was not any apparent dose effect in the percentage of luminal stenosis among group 1-4 (0.14, 0.28, 0.51, 1.11 ug/mm^2) as I acknowledge deviations considering small *N* number (Figure 3, Table 1). Albeit I found group 5 (2.22 ug/mm^2) with high dose paclitaxel most significantly inhibited stenosis in the graft, I also witnessed many cyst formations which informs us there was poor tissue affinity to the outer graft surface. In Figure 6, surrounding tissue of the graft was stained as a mean to differentiate cellular phenotypes with respective antibodies and Table 2 shows the presence of different cell types. Since smooth muscle cells possess both proteins, α-

smooth muscle actin (α-SMA) and desmine, they appeared to be α-SMA-positive/desmine-positive. Likewise, I may designate myofibroblasts and fibroblasts to be α-SMA-positive/vimentin-positive and vimentin-positive respectively. I found predominant cellular phenotypes of myofibroblasts and fibroblasts rather than smooth muscle cells in control group whereas increased number of smooth muscle cells and myofibroblasts were present in group 2 (0.28 ug/mm^2). In group 3 (0.51 ug/mm^2) and above, I found very weak staining and loss of detection in all cellular phenotypes owing to high level of paclitaxel [26]. It should be noted that the data in Figure 2 should be not correlated to this result. When I see the release pattern in Figure 2, it seems the trend does not depend on the concentrations of paclitaxel such that I caught in Figure 6 because it is not biologically meaningful. Nevertheless, it allows us to understand the *in vitro* release pattern merely illustrates the burst release of paclitaxel in the beginning and the sustained release over a period of time. Though in reality, paclitaxel release happens fairly quickly during a systemic circulation and after a 6-week- period of incubation *in vivo*, almost all paclitaxel coated on the graft was released (Table 3) [32]. Considering that, I speculate weak staining of group 3 and above (albeit not shown) is due to a foreign body response elicited by high concentration of paclitaxel. Vascular repair initially requires neointima formation and mostly smooth muscle cells play a major role in early thickening of neointima and in an acute injury, even many smooth muscle-like cells can be found. In striking similarity, myofibroblasts are regarded as

a key factor in wound healing process for their mechanical ability to provide contractibility and synthetic properties forming fibrosis. During the process, they migrate to the graft to deliver a contractile force suturing graft and tissue together for reconstruction [17, 20]. Furthermore, adventitial fibroblasts also take part in the process as they go through phenotypic modulation to myofibroblasts upon vascular injury [17, 33]. There is no surprise finding these cellular phenotypes in intima and in graft surrounding tissue and it explains why there was predominant presence of myofibroblasts and fibroblasts in the surrounding tissue of the control group as SMCs mostly reside in neointimal tissue for the aforementioned role of each phenotype. High dose paclitaxel release from the outer surface of the graft as well as the interior, however, impeded cell migration and infiltration onto the either surface [14, 34]. Cellular adherence is thought to be one of the key steps in wound healing process and in the absence of rapid myofibroblasts infiltration, conspicuously few number of cells can exist on the outer graft, and it elongates exposure time of a bare graft triggering foreign body reactions [16, 18, 19]. Based on a time period, there are two types of inflammatory responses that can occur, comprising an acute phase in the beginning and a chronic phase subsequently [18, 35]. Inflammatory phases largely depend on the size or degree of tissue injury. During the acute phase, from only hours to days, provisional matrix is formed and the wound site is cleaned up while fluid/proteins exudation and neutrophil attack occur. When excess amount of blood runs into the wound site as blood vessels dilate, a number of

blood/tissue proteins are released and leukocytes infiltrate the scarred region, followed by differentiation of monocytes into macrophages. If the body still senses the presence of foreign material until then, inflammation stage is transferred to a next chronic phase during which blood vessels proliferate and connective tissue restructures itself in the presence of macrophages and lymphocytes [18-20]. Tissue granulation begins involving migration and proliferation of fibroblasts, and subsequent capillary formation. In the final step, a fibrous capsule is developed and it is often referred as a cyst [18, 19, 36]. In this work, a few cyst formations were observed in group 3 and above. Especially group 5 involved 2 grafts completely separated from the surrounding tissue and the result is in agreement with the aforementioned weak staining. In sum, relatively low dose paclitaxel coated graft could markedly alter intimal response interior but high concentration of paclitaxel in return, displayed an adverse foreign body response [28]. As histological findings showed, we would like to stress the point that low amount of paclitaxel coating on the graft greatly can enhance the level of patency and graft thrill, and it also can minimize a foreign body reaction.

5. CONCLUSION

Herein, I demonstrated high concentration of paclitaxel coated ePTFE AV graft reduces stenosis caused by neointimal hyperplasia but it also promotes severe foreign body responses and tissue granulations leading to a cyst formation. Therefore, in order to achieve an optimal condition balancing two components, stenosis and a foreign body response, the relationship between the amount of an anti-proliferative agent released from the biomaterial and the severity of inflammation must be uncovered and accounted in prior to a clinical trial.

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7. Figure

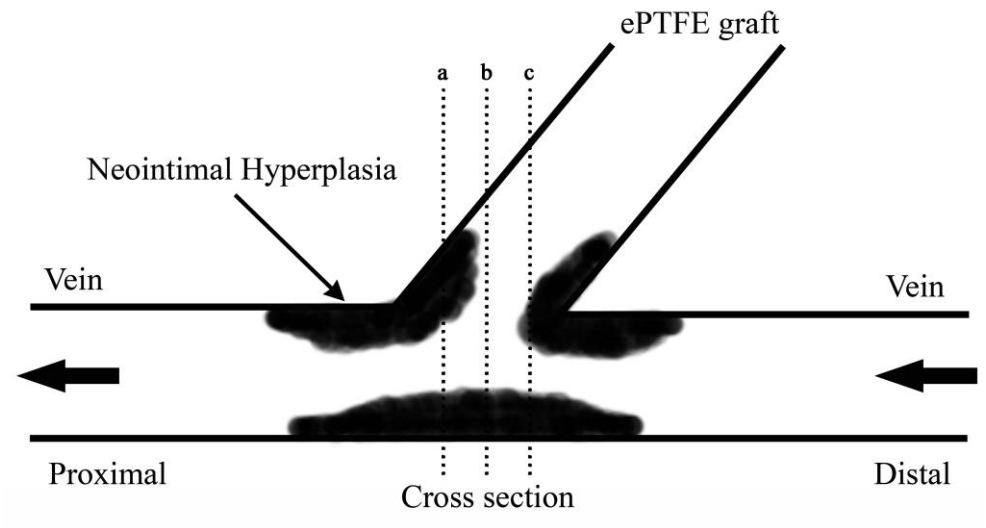

Figure 1. Diagrams of a graft-venous anastomosis. (A) Serial sections were taken to obtain cross sections around the center of the graft-venous anastomosis, and three of these sections were analyzed to find the mean percentage of luminal stenosis. One section was obtained at the center of the anastomosis; the others were obtained 2 mm to the proximal side and 2 mm to the distal side from the center of the anastomosis. (B) A cross-section was taken in the center of the anastomosis. The grey area indicates neointimal hyperplasia in the lumen of the venous anastomosis.

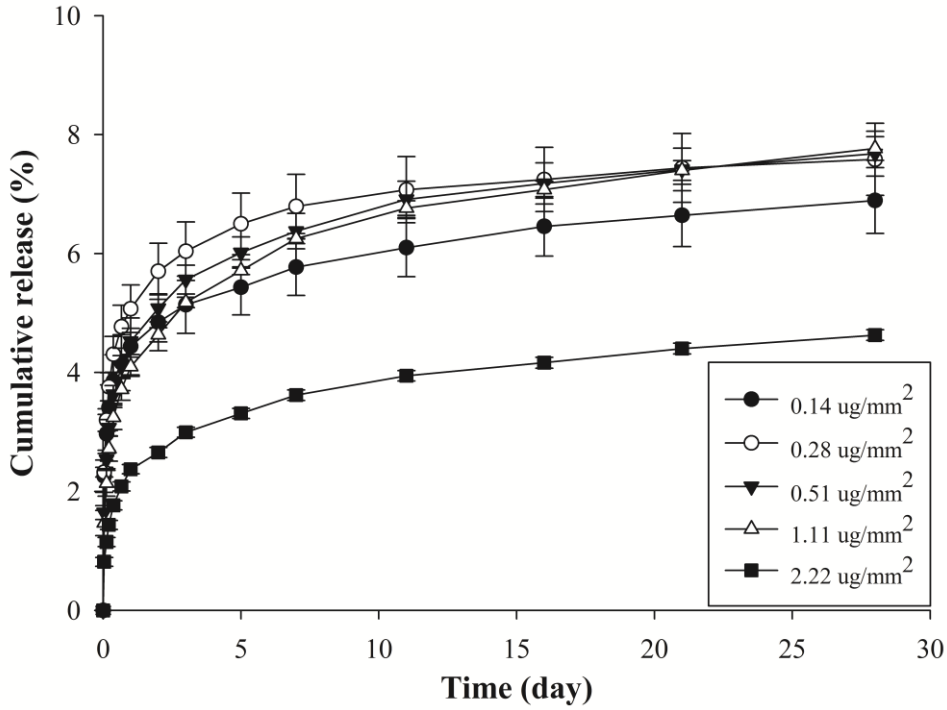

Figure 2. Cumulative in vitro release profiles from ePTFE grafts containing 0.14, 0.28, 0.51, 1.11 and 2.22 ug/mm^2 paclitaxel. Data are the means of five experiments and bars represent standard errors.

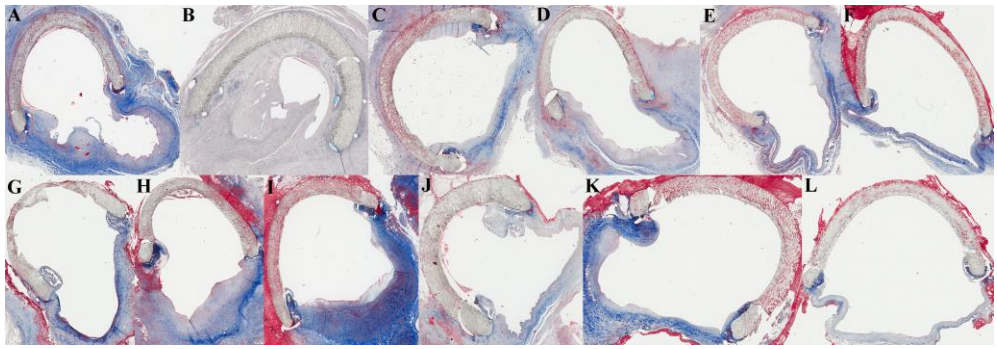

Figure 3. Representative cross sections of graft-venous anastomosis from control grafts (A-B), and from paclitaxel-coated grafts of group 1 (C-D), group 2 (E-F), group 3 (G-H), group 4 (I-J) and group 5 (K-L), respectively. (Masson trichrome stain) Neointima can be recognized as pale blue stained area in the cross section.

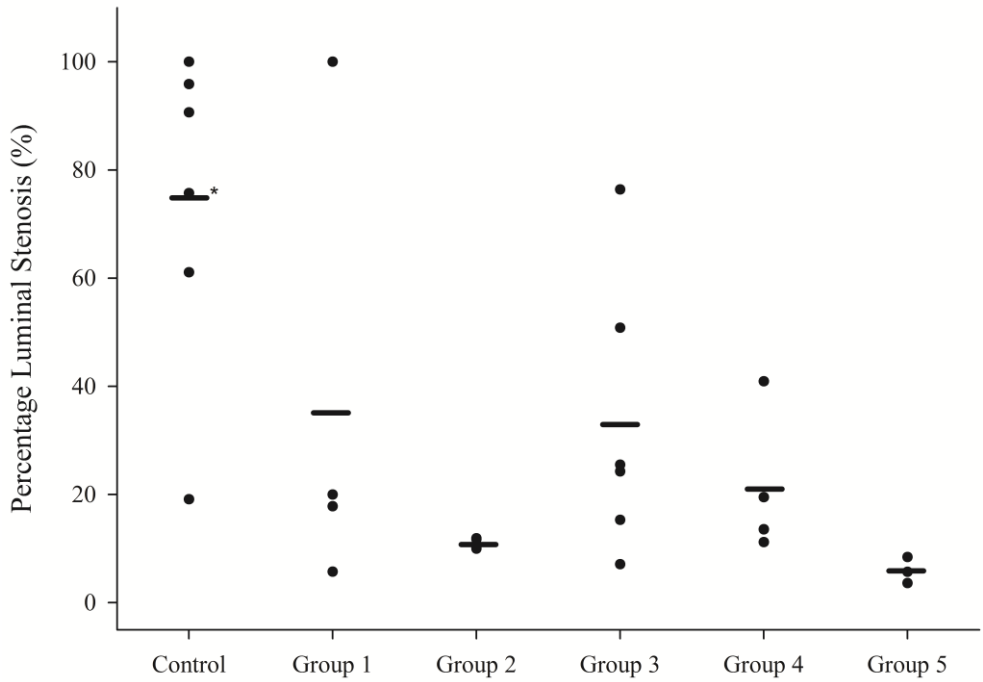

Figure 4. Comparison of the percentage luminal stenosis among the control (n=6) and the paclitaxel-coated groups. * indicates the mean value of each group. Note that the differences between the control and paclitaxel-coated group 2 and 5 were statistically significant ($P<0.05$ with unpaired Student's *t* test).

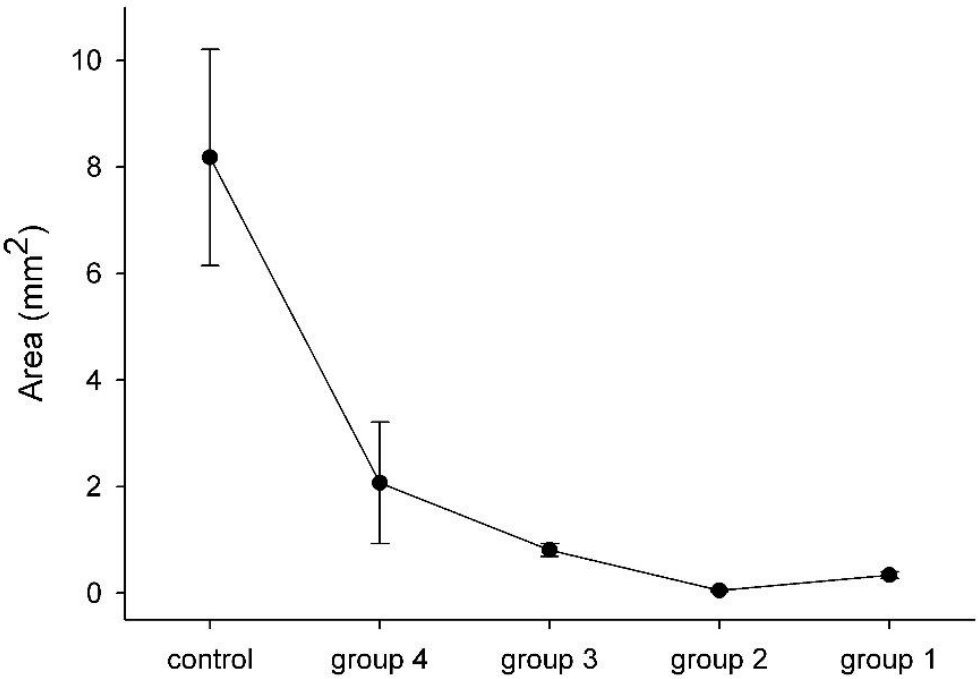

Figure 5. Comparison of luminal stenosis area (mm^2). A decreasing trend from control to group 2 and a slight incerease from group 2 to group 1 is observed.

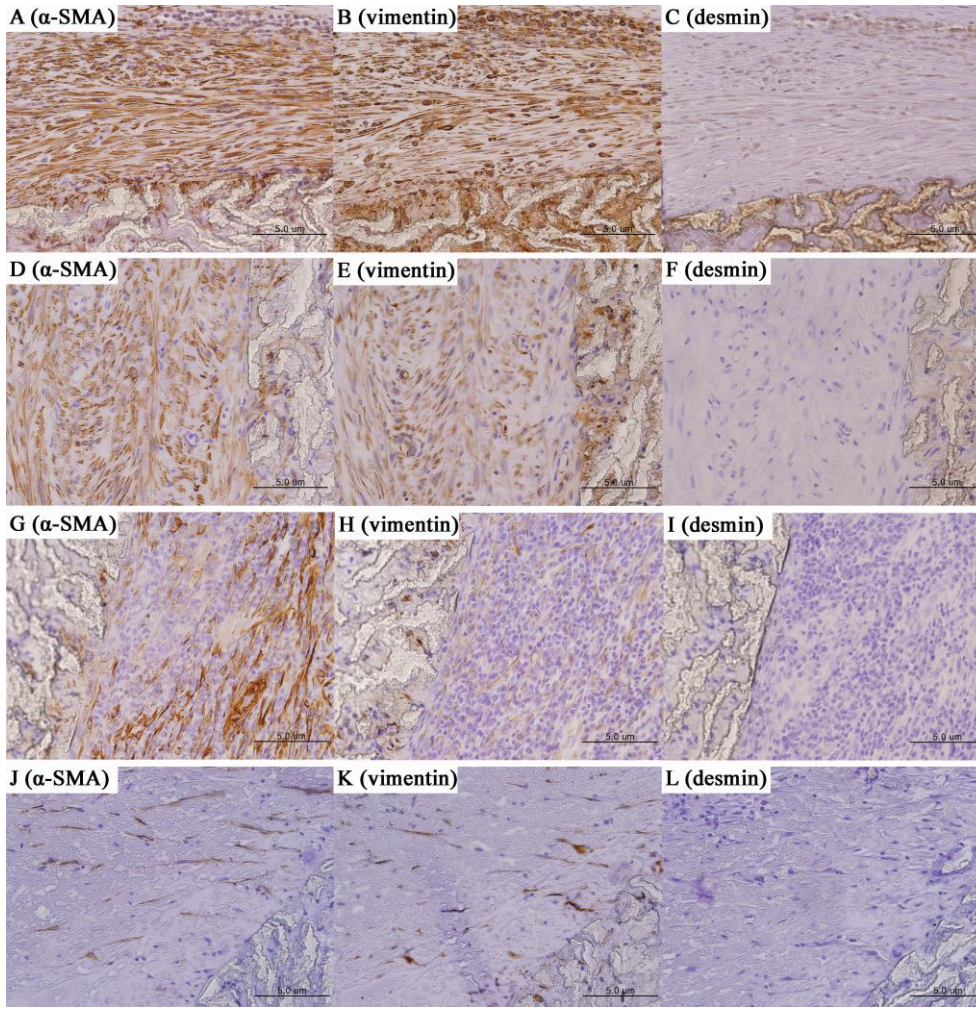

Figure 6. The cellular phenotypes of surrounding tissue outside the grafts. The slide images show sequential sections of control (A-C), and paclitaxel-coated grafts of group 1 (D-F), group 2 (G-I) and group 3 (J-L) immunohistochemically stained for alpha-SMA (A, D, G, J), vimentin (B, E, H, K) and desmin (C, F, I, L). (X400 magnification)

8. Tables

Group	Percentage Luminal Stenosis (%)	Patency	P^a
Control	76.7 ± 33.5	1 / 6	
Group 1 ($0.14\ ug/mm^2$)	14.5 ± 4.4	3 / 4	> 0.05
Group 2 ($0.28\ ug/mm^2$)	10.9 ± 0.5	4 / 4	0.004
Group 3 ($0.51\ ug/mm^2$)	18.0 ± 4.3	4 / 4	> 0.05
Group 4 ($1.11\ ug/mm^2$)	21.3 ± 6.8	4 / 4	> 0.05
Group 5 ($2.22\ ug/mm^2$)	5.9 ± 1.4	3 / 3	< 0.001

Table 1. Morphometric data at the graft-venous anastomosis. The percentage of luminal stenosis is presented as mean ± standard error. Patency indicates the ratio of cross-sections which percentage luminal stenosis was less than 50%. P^a vs control

	α-SMA	Vimentin	Desmin
Smooth muscle cells	+	-	+
Myofibroblasts	+	+	-
Fibroblasts	-	+	-

Table 2. Determining the cellular phenotypes through immunohistochemistry. If a cellular phenotype shows a positive reactions towards the respective antibody of the protein: α-SMA, Vimentin, or Desmin.

sample	area	amount(㎍)	Length (mm)	amount(㎍/㎟)	% Release
Group 1 (0.14ug/mm^2)	2.6	2.39	65.0	0.0020	99
Group 5 (2.22 ug/mm^2)	55.3	41.08	60.0	0.0400	98

Table 3. Amount of paclitaxel remaining in the graft after a 6-week-observation. One graft from each group 1 and 5 was used for analyzing the remaining amount of paclitaxel.

Group	Name	Inflammation	Observations	Patency	Note
group 1	DE1		No thrilling, good blood flow, clear body fluid	O	
	DE2		occluded	X	thrombosis
	DE3		distal part of the vein interrupted blood flow	O	
	DE4		Good blood flow	O	
group 2	DQ1	○	Unreasonably thin artery and vein, swelling immediately after the surgery, autopsy revealed vein suture site causing copious bleeding		excluded
	DQ2	○	autopsy revealed vein suture site causing copious bleeding		excluded

DQ3	○	Due to severe swelling, earlier sacrifice was performed. Severe hematoma was observed. Vein was sutured incompletely.		excluded
DQ4	×		O	
DQ5	×	Partially slight hematoma was observed	O	
DQ6	×	Good thrilling and blood flow. Slight hematoma was formed	O	
DQ7	×	Good thrilling	O	
DQ8	○	Severe swelling and inflammation. Early sacrifice was performed		excluded

	DH1	×	Slight swelling	O	
	DH2	○	Incomplete suturing		excluded
	DH3	○	Slight inflammation	O	
	DH4	○	Severe swelling, dead. Hematoma was observed. Vein suturing burst.		excluded
group 3	DH5		Drain was cut during surgery		excluded
	DH6		Cyst formation	X	
	DH7			O	
	DH8			O	
	DH9		Graft was twisted. Body fluid was pulled out of the graft due to pressure.		excluded
group 4	DT1			O	
	DT2		Good blood flow	O	
	DT3		Good blood flow. Thrombosis occurred during sampling. Thrombus excluded	O	

	DT4		O
	DT5	Incomplete vein suture	excluded
	DT6	Euthanasia	excluded
group 5	DF1	Great blood flow and thrilling	
	DF2	Great blood flow and thrilling, cyst formation	
	DF3	Great blood flow and thrilling, cyst formation	

Table 4. Supplementary information on animal experiments

Abstract in Korean (국문 초록)

신장 투석 환자들의 혈관 확보를 위해 동정맥루 (Arteriovenous graft)가 사용되는데 문합 부위를 중심으로 1~2 년 내로 발생하는 위내막 과다형성 (Neointimal hyperplasia)에 의한 협착증 때문에 혈관이 막히게 된다. 이를 방지하기 위해 위내막을 주로 형성하는 평활근 세포들 (smooth muscle cells)의 증식을 억제시키는 파클리탁셀이 널리 이용되고 돼지 실험을 통해 파클리탁셀이 코팅된 인공혈관이 위내막 형성을 줄이고 높은 개통율에 기여한다는 연구 결과가 있다. 위 논문에서는 여러 가지 코팅 방법 중 비용, 시간, 균일성에 용이한 담금법 (dipping method)을 사용 한다. 담금법을 사용하면 인공혈관의 양 면에 파클리탁셀이 고루 도포되는데 바깥면에 코팅된 파클리탁셀은 고유의 항증식성 성질로 혈관과 주변 조직 결합을 낮추어 이물 반응을 일으킬 수 있고 심한 경우 혈관 주위로 낭종 (Cyst)이 형성되어 주변 조직과 완전히 분리되게 된다. 이러한 이물 반응을 줄이고 개통율을 높이기 위해 위 논문에서는 아래와 같은 농도; [Control (0 ug/mm^2), Group1 (0.14ug/mm^2), Group2 (0.28ug/mm^2), Group3 (0.51ug/mm^2), Group4 (1.11ug/mm^2), Group5 (2.22ug/mm^2)]의

파클리탁셀로 코팅된 인공혈관을 이용하여 농도 최적화를 시도하였고, 그 결과 컨트롤은 심한 협착으로 혈관 막힘이 보였으며, 0.14ug/mm^2 에서는 75%의 개통율을, 0.28ug/mm^2 이상에서 100%의 개통율과 낮은 위내막 형성이 관찰되었지만 0.51ug/mm^2 이상부터 이물반응으로 인한 감염과 낭종이 발견되었다. 그러므로 낮은 농도 (0.28ug/mm^2)로 담금법 코팅을 할 경우 주변 조직과 혈관의 결합에 해를 가하지 않고 이물반응을 일으키지 않는 선에서 위내막 형성을 최소화하여 개통율을 높일 수 있다.

주요어: 파클리탁셀, 동정맥루, 위내막 과다형성, 이물반응, 개통율, 신장 투석

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