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# Biosynthetic bacterial cellulose graft as arteriovenous fistula – a complement to existing synthetic grafts?

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**ABSTRACT**

**Introduction:** There is an increasing need for vascular prosthesis in dialysis medicine for the construction of arteriovenous (AV)-fistulas for dialysis access. The aims of this study were; a) to develop and validate a new experimental AV-fistula model for larger animals using grafts of bacterial cellulose (BC) between the common carotid artery and the external jugular vein; b) to observe the immediate and intermediate properties (macroscopic and angiographic patency and the macro- and micro thrombogenicity) of the grafts.

**Materials and Methods:** As graftmaterial bacterial cellulose was used, produced around a preformed scaffold. Bacterial cellulose (BC) is a material produced by the bacteria acetobacter xylinum. A pilotstudy was conducted on 6 pigs to validate the animalmodel and the new graftmaterial. In the following survival study a BC-graft AV-fistula was constructed in 15 pigs.

**Results:** In the pilot study, 5 out of 6 animals had a patent AV-fistula 4 hours after implantation. In the survival study, after 4 (n3) and 8 (n10) weeks an angiography was performed prior to explantation of the BC-graft. All grafts were occluded with a presumed platelet plug. We conducted an additional acute patch-test comparing the BC and expanded PolyTetraFluoro-Ethylene. A patch of BC and ePTFE was applied to the right and left common femoral artery respectively. At explantation three hours later, all BC-patches showed a thin gel like layer, most likely consisting of platelets, throughout the whole surface while the ePTFE-patch showed no, or minimal, signs of platelet adhesions.

**Conclusion:** Theoretically the cellulose might be similar to autologous veins considering risk of infections and thrombogenicity. The animal model and the graft material showed good potential in the pilot study. The survival study was discouraging with the reason for occlusion still to be explained. Bacterial cellulose has a good potential but further development and studies need to be performed.

***Key words:*** *Bacterial cellulose, animal model, AV-fistula, arteriovenous fistula, acetobacter xylinum*

## Introduction

Vascular grafts are used in a variety of bypass surgeries on the peripheral arteries and the coronaries. In addition, there is an increasing need for vascular prosthesis in dialysis medicine for the construction of arteriovenous (AV)-fistulas for dialysis access. It is a well-known fact that autologous veins in this setting are overall better than synthetic vascular prostheses [1]. Compared to prosthetic grafts, autologous veins have a lower risk of thrombosis, stenosis and infection [1].

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Hence, autologous veins are used as much as possible, but because this is a limited resource, there is a regular reliance on prostheses made of polyethyleneterephtalate (PET) and expanded polytetrafluoroethylene (ePTFE).

Bacterial cellulose (BC) is a material produced by the bacteria, acetobacter xylinum. It is a polysaccharide with a range of desirable properties. BC has high mechanical strength and compliance that is similar to native arteries [2,3]. Furthermore, earlier experimental in vitro studies have shown low thrombogenic activity comparable to PET and ePTFE [4].

The aims of this study were: a) to develop and validate a new experimental AV-fistula model for larger animals; and b) to observe the immediate and intermediate properties (macroscopic and angiographic patency and the macro- and micro thrombogenicity) of the BC grafts.

## Materials and Methods

### *Animals*

A pilot study was initially conducted on five pigs with a median weight of 45 kg (41-47).

In the main study that followed, called the survival study, fifteen pigs were used that weighed between 25 and 31 kg.

All animals received care and all experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals " (revised by NIH 1996). The ethical committee at the Faculty of Medicine, Lund University, Sweden, approved the research protocol (M126/11).

### *Graft*

The tubes of bacterial cellulose were produced with a method previously described [5]. In short, bacterial cellulose is created by the bacterium, Gluconacetobacter xylinum (BPR2001, ATCC 1700178, American Type Culture Collection). The cellulose is deposited on oxygen permeable silicone tubes (4 mm in outer diameter, Silicon PE4032. Optima Scandinavia AB, Sweden) submerged in a special nutrient medium (Table 1).

By varying oxygen pressure by 0.10-0.15 Psi (technical air with 21% oxygen ratio AGA AB, Sweden) of the silicon tube along with the growing time, the quality of the cellulose tubes can be altered [5]. The tubes used in this experiment were fermented for 6 and 14 days with oxygen pressure of 0.15 and 0.10 Psi, respectively.

After fermentation, the BC tubes were purified by repeated boiling in 0.1 M NaOH and then repeated boiling in Millipore™ Water (Merck Millipore, Darmstadt, Germany). Afterwards, they were steam sterilized (for 20 min (121° C).

## Surgical Procedure

### *Pilot study-AV-fistula model development*

### *Primary surgery*

All five pigs were anesthetized using induction with Ketaminol vet®. (Intervet International B.V., the Netherlands) and Midazolam® (Panpharma S.A. France). The anaesthesia was maintained using continuous infusion of Propofol® (B. Braun Melsungen AG, Deutchland), while intravenous Fentanyl® (B. Braun Melsungen AG,

**Table 1.** Compounds of special nutrient medium.

| Culture Medium | | Trace Metal Solution | | Vitamin Solution | |
|---|---|---|---|---|---|
| **Compounds** | **Amount** | **Compounds** | **Amount** | **Compounds** | **Amount** |
| D-Fructose | 70.00 g | EDTA | 3.00 g | Vitamin B8 (Inositol) | 0.40000 g |
| Yeast Exctract | 10.00 g | CaCl2•2H2O | 1.470 g | Vitamin B6 (Pyrodoxine HCL) | 0.08000 g |
| (NH4)2SO4 | 3.30 g | FeSO4•7H2O | 0.360 g | Vitamin B3 (Niacin) | 0.08000 g |
| KH2PO4 | 1.00 g | Na2MoO4•2H2O | 0.242 g | Vitamin B1 (Thiamine HCL) | 0.08000 g |
| MgSO4.7H2O | 0.25 g | ZnSO4•7H2O | 0.173 g | Vitamin B10 (p-Aminobenzoic Acid) | 0.04000 g |
| Trace Metal Solution | 10 ml | MnSO4•5H2O | 0.139 g | Vitamin B5 (D-Pantothenic Acid) | 0.04000 g |
| Vitamin Solution | 5 ml | CuSO4•5H2O | 0.005 g | Vitamin B2 (Riboflavin) | 0.04000 g |
| Purified Water | Up to 1L | Purified water | Up tp 1L | Vitamin B7 (D-Biotin) | 0.00040 g |
| | | | | Vitamin B9 (Folic Acid) | 0.00004 g |

Deutchland) was used for analgesia. The pigs were intubated and ventilated with a mix of air and oxygen (30 / 70%). One preoperative dose of antibiotics, Streptocillin Vet® (Boehringer Ingelheim Vetmedica, Sweden), was administered. During the procedure, pulse rate and pulse oxymetry were monitored.

Using a standard vascular surgical technique, a longitudinal skin incision parallel to the midline was made in proximity to the sternocleidomastoid muscle. The muscle was exposed and fully mobilized. The dorsomedial part of the muscle was divided to minimise the risk of kinking the graft. The common carotid artery and the external jugular vein were exposed unilaterally (right side). In the first three animals, the grafts were implanted bilaterally. The animals were heparinised with 5000 IU of standard Heparin (LEO Pharma AB, Sweden) intravenously prior to clamping. The distal part of the jugular vein was ligated to avoid risk of excessive retrograde blood flow to the head. After clamping the artery, a longitudinal incision was made 7-10 mm in length in the proximal part of the artery, and a similar incision was also made in the distal part of the vein. A BC graft, ranging between 30-60 mm of length, was anastomosed using uninterrupted 6-0 Prolene®(Ethicon) sutures to the artery and vein, respectively. The clamps were released and blood flow restored (Figure 1). The patency of the graft was assessed using a flow meter (MEDISTIM CardioMed CM-1005 Flow Meter, Norway) and palpation of murmur.

The animals were kept anesthetized for two hours prior to either graft explantation and euthanasia using Alfatal ® (Omnidea AB, Sweden) (n3) or wound closure (n2) utilizing resorbable sutures subcutaneously and intracutaneously and thereafter awakened and transferred to the local experimental recovery facility.

During the two-hour observation time, continuous (every 30 min) measurement of activated clotting time (ACT, Figure 2) and blood flow in the AV-fistula was performed. When ACT was less than 150 sec, another dose of standard heparin (2500-3000 IU) was administered.

In the surviving two animals, 5000 anti-FXa U of low molecular weight heparin (LMWH, Fragmin® Pfizer AB, Sweden) was injected subcutaneously once daily for five days. These two animals were cared for at the local experimental facility for nine days.

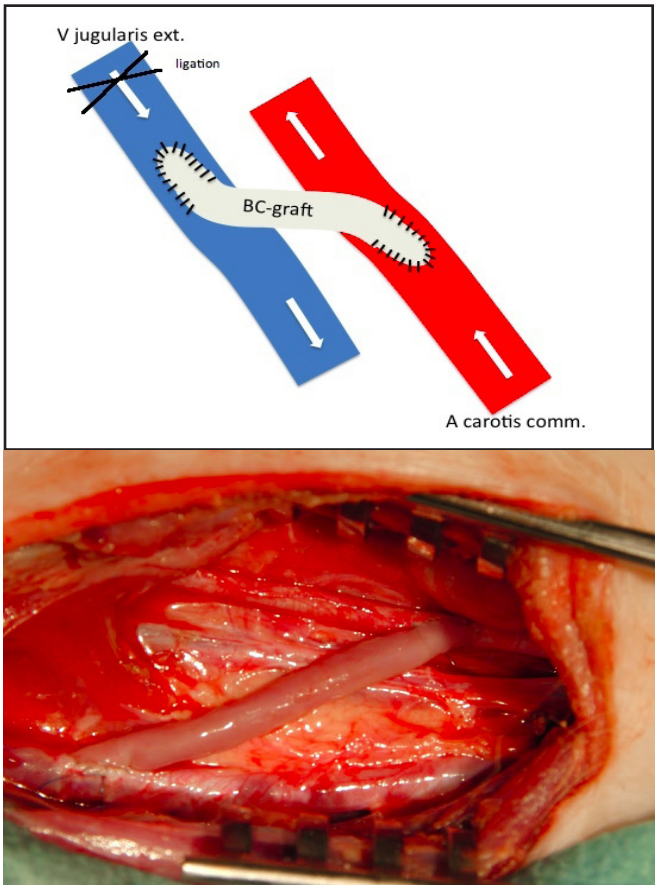



**Figure 1.** Schematic drawing and photo of AV-fistula between common carotid artery and external jugular vein.

***Secondary surgery/Explantation***

In three animals, the grafts were explanted 2 hours after primary surgery and assessed for macroscopic occlusion and flow. The animals were then euthanized. The remaining two animals were anesthetized, nine days after primary surgery, in the manner described earlier. No post-operative complications were seen and the weight gain was 4 and 5kg, respectively. The grafts were dissected and blood flow and diameter was measured prior to explantation and euthanization.

***Survival study***

***Primary surgery***

Anaesthesia, vessel exposure and wound closure was carried out in the same manner as in the pilot study previously described. The BC grafts implanted had a length ranging between 40-65mm.

For the initial two to three days, post-operative animals were observed and cared for at the local experimental facility and administered 5000 anti-FXa U of LMHW (Fragmin® Pfizer AB, Sweden) once daily for the first two days. The animals were then returned to the breeder and stayed there for one or two months.

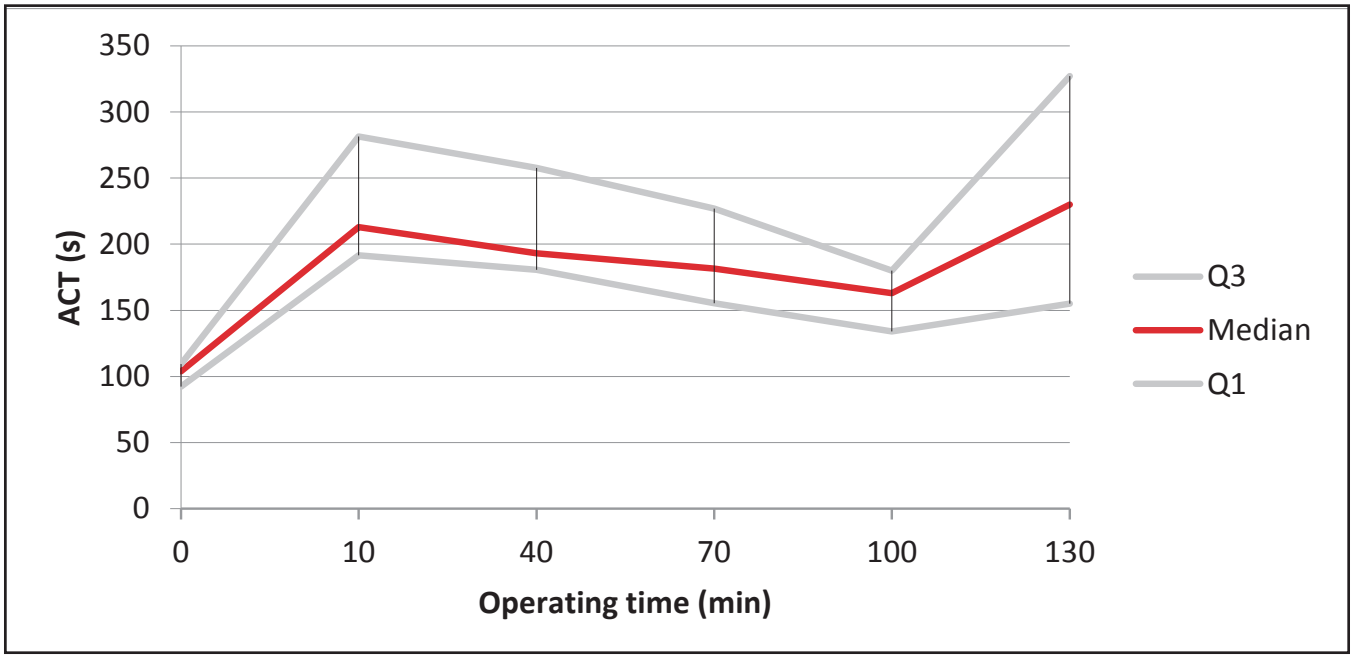


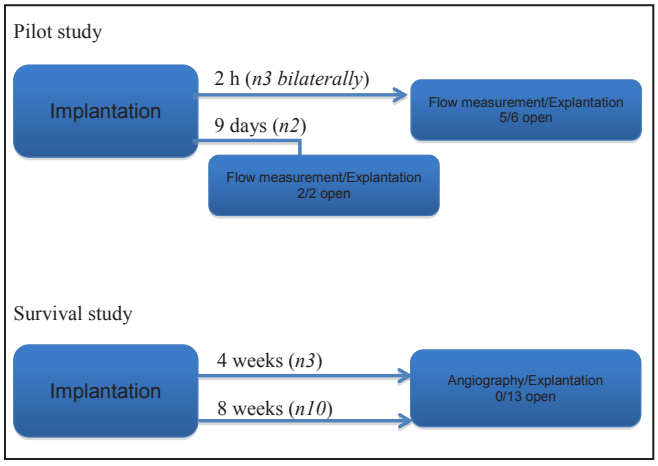

**Figure 2.** Activated Clotting Time (ACT) in pilot study. First (Q1) and third quartile (Q3) in grey.

**Secondary surgery/explantation**

The second and final operation was performed after one (n3) and two (n10) months. Anaesthesia was performed as described above.

The common-, superficial- and deep femoral artery was exposed in the left groin. With the Seldinger technique, a short standard 10F introducer was placed in the common femoral artery. 5000 IU of standard Heparin (LEO Pharma AB, Sweden) was administered intravenously. Under fluoroscopy (Philips Veradius Neo, Philips Healthcare, The Netherlands) a catheter was selectively placed in the proximal part of the right common carotid artery and an angiogram was performed (Omnipaque, GE Healthcare, USA).

After angiography, the BC grafts were explanted and snap frozen using dry ice in ethanol (70%). The animals were then euthanized.




As part of evaluating the thrombogenicity of the BC material, an acute test was conducted comparing BC and ePTFE on four animals before euthanization. The common-, superficial- and deep- femoral arteries were exposed in the right groin. Before clamping the vessels, an extra dose of 3000 IU standard Heparin was administered intravenously and blood flow was measured. A longitudinal 13-20mm long incision in the common femoral artery was made on both sides. A patch of BC and heparinized expanded PolyTetraFluoroEthylene (GORE-TEX PROPATEN Vascular Graft; W. L. Gore & Assoc, Flagstaff, Ariz) was sutured using uninterrupted 6-0 Prolene® (Ethicon, UK) on the right and left side, respectively. Blood flow was restored. After three hours, the blood flow was measured and the patch was explanted.

**Results**

***Pilot study – AV-fistula model development***

***Primary surgery/implantation***

On three pigs, the BC grafts were implanted bilaterally. All six grafts were observed for two hours. Of these six grafts, one occluded shortly after blood flow was restored. One graft ruptured at the suture line at the arterial anastomosis just before the two hours had elapsed but was open and functioning until then and is included in the presented data. The remaining four grafts had normal functioning. Median operating time for both sides was 310 minutes (range 305-390 min).

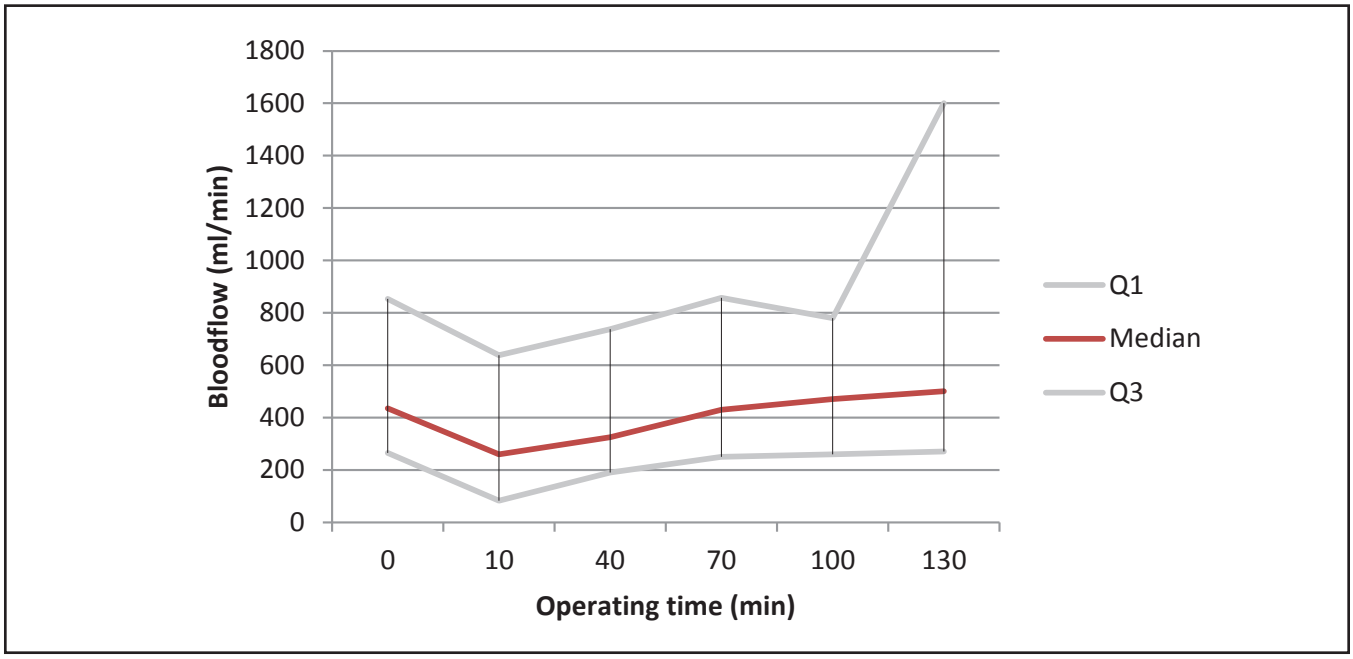



**Figure 3.** Median interquartile range of AV-fistula blood flow. First (Q1) and third quartile (Q3) in grey.

Median time for artery clamping was 60 minutes (range 45-65 minutes). The ACT was continuously measured (Hemocron Jr®, ITC Medical, USA).

Results of the blood flow measurements are depicted in Figure 3. One of the grafts (nr 3) occluded somewhere between 10 and 40 minutes. Two grafts (nr 4,5) showed decreasing blood flow after 10 minutes. Gentle manipulation of the graft and repositioning of the probe restored blood flow to ordinary levels.

**Secondary surgery/Explantation**

The two remaining animals were anesthetized and the grafts explanted on the ninth post-operative day, as described earlier. Blood flow was measured at > 200 ml/min in both grafts. After explantation, the grafts were examined macroscopically. There was a thin greyish granular layer of what were believed to be platelets in the vicinity of the anastomoses.

**Survival study**

**Primary surgery/Implantation**

Median operating time was 90 minutes (min 65/max 165 min). Median time for artery clamping was 51 minutes (min 30/max 116). A palpable murmur at the site of the AV-fistula was present in 11 out of 15 animals. Perioperative flow measurements were carried out over the AV-fistula with a median flow of 390 ml/min (min 70/max 670).

Two animals died - one within the first two post-operative hours, most likely the result of respiratory problems as the autopsy showed no signs of bleeding. The second animal died two days after surgery and the autopsy revealed major bleeding in the neck based on rupture of the arterial anastomosis.

**Secondary surgery/Explantation**

During follow up, none of the remaining animals showed any signs of physiological or neurological impairment. They were monitored by clinical evaluation and no analysis of blood plasma was carried out. The wounds healed without any signs of infection. The animals had normal weight gain and showed no signs of fever.

At 1 month (n3) and 2 month (n10), the animals underwent secondary surgery as described previously. The angiography showed occlusion of all grafts (Figure 4).

At explantation, profound external scar tissue was observed that indicated some sort of host versus graft reaction. There were no macroscopic signs of infection. All grafts were occluded with a presumed platelet plug (Figure 5.). The anastomoses showed no signs of technical or surgical malfunction. They had a wide lumen with no stenosis, with both artery and vein being unaffected, open and had no visible dissection or hematoma.

***Patch trial***

The patches were explanted, including 6mm of the adjacent vessel wall, and assessed visually. All BC grafts exhibited a thin layer most likely consisting of platelets throughout the whole surface (vessel unaffected) whereas the ePTFE grafts showed minimal amounts of

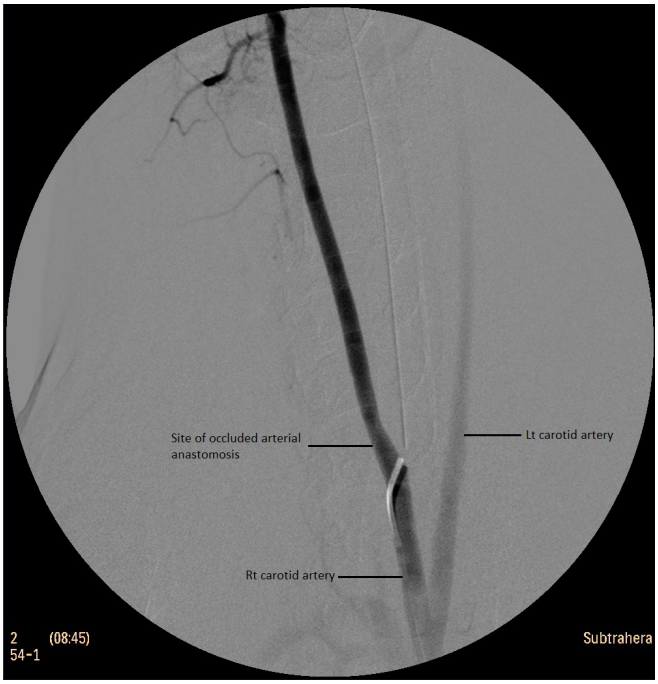



**Figure 4.** Angiography of the right common carotid artery at followup.

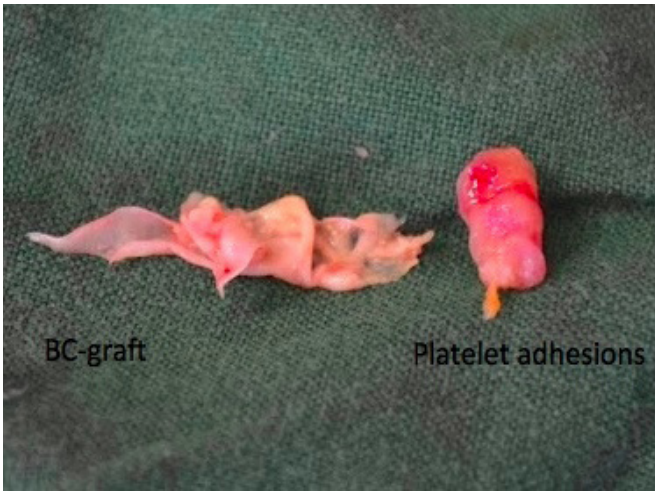



**Figure 5.** Dissected and opened BC graft (left) and extracted suspected platelet plug (right).

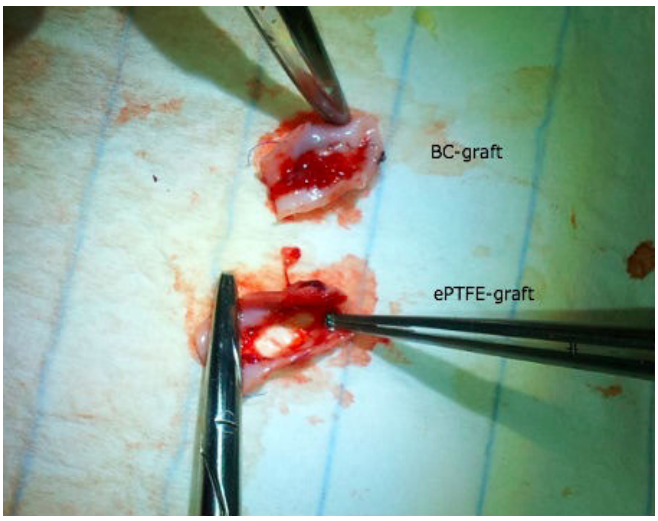



**Figure 6.** Dissected common femoral artery with BC patch and ePTFE patch. The BC graft has a thin layer of thrombocyteadhesion.

platelet adhesions close to the suture line (Figure 6). All vessels were open and patent.

**Discussion**

When constructing AV-fistulas, one finally reaches the point when they run out of autologous veins and there is a need for a synthetic alternative. Previous in vitro studies of bacterial cellulose revealed low thrombogenic properties [4] and investigations performed on sheep have shown a possible patency for the BC graft of up to 13 months [6].

In the pilot study, promising results were found nine days after implantation. The grafts were open and had small amounts of thrombocyte adhesions. Conversely, the results from the survival study were discouraging. The reasons for the occlusions have yet to be explained but there are a few factors to take into account.

For the pilot study, a three days longer postoperative administration of LMWH was used compared to the survival study (five vs. two days). This is less likely to be of importance, as LMWH is known to have little impact on the platelets and these observations point towards the activated platelets having a major contributing affect in the occlusions. However, other studies using LMWH during the maturation process, such as 60 days in AV-fistulas constructed on paediatric haemodialysis patients, indicate a positive effect on lowering the risk of early thrombosis with prolonged LMWH administration [7]. Whether this 60-day period of LMWH treatment is applicable here with a difference of three days between the groups is questionable.

No thrombocyte aggregation inhibitors were used in either study – would doing so have improved the outcome? Earlier studies imply this [8, 9]. However, this cannot explain the difference between the pilot study and the survival study as there was no antiplatelet therapy in either case. Macroscopically, a clear difference was noticed between the ePTFE patches and the BC patches, where the latter had a substantially thicker and larger greyish granular layer of presumed platelets.

In future studies, it could be suggested to use a more aggressive thrombocyte aggregation inhibition. A combination of aspirin with one of the modern agents, such as ticagrelol or prasugrel, might improve results. In uremic humans, this might not be relevant based on their decreased platelet adhesiveness.

An in vitro test with a chandler loop setting (Figure 6) might answer some of these questions prior to new animal experiments.

Could there be a technical issue to explain the plugged grafts? When evaluating anastomosis, no signs of stenosis caused by the suture line were seen. Neither was there any sign of dissection or hematoma in the intima. Further, both the artery and vein were unaffected. The possibility of technical failure is of course a plausible explanation but this cannot explain why all grafts occluded.

The grafts used in the pilot study were of a different generation compared to those used in the survival study. The later generations were further developed for better handling, i.e. more rigid to lessen the risk of kinking. The first generations have been tested in vitro and have exhibited low thrombogenic activity [4]. The later generations used in the survival study showed a 100% occlusion rate, the occlusions having a macroscopic appearance of thrombocyte aggregations (Figure 5). Could the change in rigidity be coupled to increased thrombogenicity? Properties that need to be studied to explain the implant failure include mechanical evaluation, structural differences, differences in hemocompatibility and levels of endotoxins between the BC graft generations.

The mechanical characterization can be assessed through measuring radial tensile strength.

Differences in surface structure between the graft generations, potentially having an impact on thrombocyte adhesions, may be visualised using a scanning electron microscope (SEM).

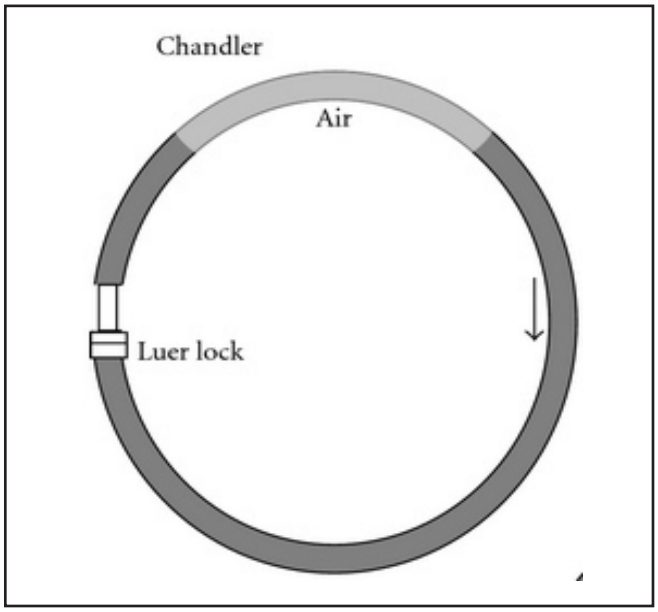



**Figure 7.** Schematic drawing of a Chandler loop.

When assessing hemocompatibility, different tests are available. One of them is the classical ex vivo modified Chandler loop model (Figure 7).

With a chandler loop, one is able to circulate whole blood and test it on different tubing materials analyzing platelet consumption, levels of thrombin-antithrombin complex as parameters of coagulation and sC3a and sC5b-9 as parameters of complement activation. This setup could be used to examine different generations of BC-graft, whole blood prepared with single thrombocyte aggregation inhibition (aspirin) or double inhibition (aspirin in combination with ticagrelol or prasugrel).

To determine the presence of endotoxins, a commercially available kit that makes use of the clotting reaction induced by limulus amebocyte lysate can be utilized. This is an extract from blood cells from the horseshoe crab. This reagent reacts with the lipopolysaccharide of the cell wall in gram-negative bacteria. High levels of endotoxins can indicate a malfunctioning purification process of the BC grafts.

These tests will indicate differences in thrombogenicity between the graft generations used. However, it will not answer if there is an undetected process that results in an occlusion between day nine and four weeks.

Is the pig an optimal animal model for testing AV-fistulas or is it too thrombogenic? Does it differ from sheep used in earlier studies? There do exist studies showing that pigs are suitable animals in this setting, such as that by Burnett et al. (2002). In this porcine model, ePTFE AV-fistulas (a. femoralis – v. femoralis) had a patency in 16 out of 18 grafts [10]. The grafts in that study were harvested after 2, 4, 7, 14 and 28 days and they used 325mg of Aspirin each day, making it difficult to directly compare it to the present work.

The authors conclude the following:

a. the current experimental AV-fistula porcine model is clearly feasible and promising, albeit further studies are needed to elucidate whether the per/post-platelet inhibition is recommended.
b. the evaluated BC graft has interesting and promising features from a strictly vascular surgical handling perspective.

c. the currently used BC graft is clearly unsuitable for AV-fistula in this setting because of its extensive thrombogenicity. Further studies (as outlined earlier) are currently underway to address this problem.

**Conflict of interest statement**

The authors have no conflicts of interest to declare.

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