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Preliminary Results of Small Arterial Substitute Performed with a New Cylindrical Biomaterial Composed of Bacterial Cellulose

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Abstract *Objective:* Tissue-engineered blood vessels (TEBVs) represent an innovative approach for overcoming reconstructive problems associated with extended vascular diseases by providing small-calibre vascular grafts. This study aimed to evaluate a novel biomaterial of bacterially synthesised cellulose (BC) as a potential scaffold for TEBV.
Methods: Highly crystalline cellulose was produced by a bacterium (*Acetobacter xylinum*) using glucose as a source of carbon. Using a patented process, hollow-shaped segments of BC were created with a length of 10 mm, an inner diameter of 3.0—3.7 mm and a wall thickness of 0.6—1.0 mm. These grafts were used to replace the carotid arteries of eight pigs, and after a follow-up period of 3 months, the grafts were removed and analysed, both macro- and microscopically.
Results: Seven grafts (87.5%) remained patent, whereas one graft was found to be occluded. Scanning electron microscopic examination revealed rapid re-cellularisation by recipient endothelial cells. Light microscopic examination showed a three-layered wall structure of the BC segments, with cellulose still being present in the media.
Conclusion: These data indicate that the innovative BC-engineering technique results in the production of stable vascular conduits, which exhibit attractive properties for their use in future TEBV programmes for vascular surgery.

Atherosclerotic vascular diseases, such as coronary artery disease and peripheral vascular disease, are still the largest cause of mortality in Western societies.[1] Surgical treatment of atherosclerosis began in 1952, when Voorhees et al. proposed to replace diseased blood vessels with synthetic

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fabric.[2] This led to the widespread clinical use of Dacron (polyethylene terephthalate (PET)) and Teflon (expanded polytetra-fluoroethylene (ePTFE)) grafts in vascular surgery. However, these synthetic materials are clinically unacceptable for reconstructing small-diameter arteries (i.e., <6 mm, which are required in lower extremity bypass and coronary artery bypass-grafting procedures), because they are foreign bodies and thrombosis can occur on the luminal surface, resulting in occlusion. For such small artery bypass-grafting procedures, autologous arterial or venous grafts still remain the most ideal vascular substitutes. However, they cannot always be used because of their poor quality and inadequate size or length. Moreover, a second surgical procedure is required to obtain the vessel. Thus, it is imperative to develop innovative technologies targeting the fabrication of small-calibre vascular grafts. The concept of tissue engineering using biodegradable materials was proposed recently,[3] and such materials have been developed and applied clinically with autologous cell seeding or bioreactor culture.[4–6] However, the utility of these materials in the reconstruction of small-calibre vascular grafts has not been demonstrated. Moreover, pretreatment with autologous cell seeding or bioreactor culture can be complicated and invasive and will render the graft susceptible to infection.

Bacterial cellulose (BC) is a polysaccharide that is excreted extracellularly by the *Acetobacter xylinum* bacteria as long non-aggregated nanofibrils. BC displays many unique properties, including high mechanical strength, water content, crystallinity as well as an ultrafine, highly pure, nanofibril network structure.[7] *A. xylinum* constructs a pellicle of BC that has a dense outer surface and a gelatinous layer inner surface. BC has been used in a microvessel endoprothesis[8] and as a temporary skin substitute.[9] The material has also been investigated as a scaffold for the tissue engineering of cartilage.[10] To our knowledge, BC is neither widely used nor investigated for tissue-engineering applications. This study aimed to explore the suitability of BC for use in tissue-engineered blood vessel (TEBV).

Material and Methods

Graft formation

A. xylinum produces cellulose in a highly swollen form, using glucose as the carbon source (Schramm–Hestrin medium[d]). The water-absorption capacity was between 100 and 120 times over its dry weight. Solely, one BC graft could be produced in a patent construction (Fig. 1) over a cultivation time of 10 days. The network of fine, highly entangled nanofibrils with dimensions of approximately 100 nm is seen with scanning electron microscopy (SEM) (Fig. 2). For this study, the process created moist tubes with a length of about 10 mm, an internal diameter of 3.0–3.7 mm and a wall thickness of 0.6–1.0 mm. The inner diameter could be diversified by altering the size of a glass mandrill, which was used as the shaped body wall. The BC graft consists of 1% cellulose as the microstructure and 99% water, and this allows room for cell in-growth and proliferation. To improve cell in-growth, different techniques can be used to create more free space in the BC pellicle. One method can be the use of leaching techniques, for example, introducing paraffin spheres during culture and then removing them by leaching. Another way is to use cellulases, which are enzymes that degrade cellulose and can alter the density of the BC pellicle. For our experiment, BC was purified by boiling in 0.1 M NaOH at 60 °C for 4 h and, thereafter, the material was steam sterilised (1 bar, 120 °C) for 20 min and stored in Ringer's solution until required for use.

In vivo experiments of the BC graft

Eight kinds of BC grafts were provided and implanted in juvenile, white, domestic pigs weighing between 35 and 40 kg. The study was approved by the Animal Care and Use Committee of the Friedrich–Schiller University in Jena. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals as revised by the National Institute of Health in 1985. Anaesthesia was induced and ventilatory support was established. The animals were heparinised systemically with an intravenous bolus injection of 200 units kg^{-1} and the right carotid artery was carefully prepared (Fig. 2a). Then, the target artery was resected and interposed with the BC graft using an interrupted suture with 7/0 monofilament Prolene. Haemostasis was easily obtained after the initial oozing through the graft at de-clamping. To assess the patency of the grafts, flow measurement (MediStim AS, Oslo, Norway) was carried out at a mean blood pressure of 70 mmHg (Fig. 2b). After wound closure, all animals were allowed to grow for 3 months. After this time interval, the animals were sacrificed and the implants evaluated for aspects of chronic inflammation, foreign-body responses, cell in-growth and diameter, using histology and electron microscopy (LEO 1450 VP/REM, Oberkochem-Zeiss, Germany). The native carotid artery served as the control.

Results

All pigs survived surgery. Paraplegia was not observed in any animal during the postoperative period. BC had good surgical capabilities, that is, suture and handling that were qualitatively considered to be appropriate, and flow was restored with no leaks or oozing through the polymer after the anastomoses were completed. There were no significant differences in blood flow at the proximal and distal anastomoses after the initial implantation (53.6 ml ± 10 ml and 53.7 ml ± 8.5 ml). The patency rate of all grafts was 87.5% (one of the eight grafts was found to be occluded). Although the grafts were covered by an adhesion capsule, there were no stenoses observed histologically at the proximal or distal anastomoses in any animal. There were no apparent changes such as dilatation, dehiscence or aneurysm formation in any of the grafts. There were no significant differences in the implant diameter before (3.3 mm ± 0.22 mm) and 90 days after the operation (3.2 mm ± 0.24 mm). The occluded vessel demonstrated

[d] Medium: 20 g glucose, 5 g bactopepton, 5 g barm essence, 3.4 g di-Na-hydrogenphosphat-dihydrat, 1.15 g citric acid-monohydrate per litre of pure water.

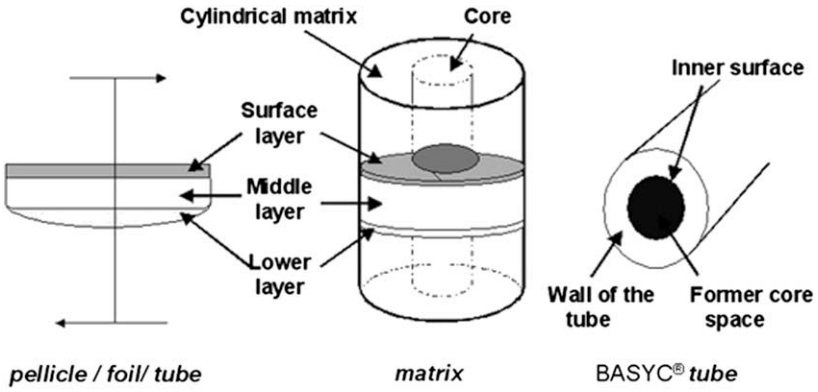

Figure 1 Assembly of BC tubes.

collapsed vascular walls. No vessel lumen could be identified, probably due to the surrounding tissue. There was no bleeding from the grafts or any formation of arteriovenous fistula.

Morphological evaluations

All patent grafts revealed no significant stenoses, histologically, and there was only a minimal intimal hyperplasia. However, the macrostructure of the BC tube appeared altered. In the haematoxylin and eosin staining, a three-layered wall construction was observed throughout the implanted BC tubes, which were nearly indistinguishable from the original carotid vessel. All grafts had a single layer of endothelium with a basement membrane and a thin layer of collagen followed by a concentric layer of smooth muscle and an outer layer of fibrous cells. Graft endothelialisation was observed at the anastomosis sites and at the centre in all the grafts. The neo-intimal thickness of the BC graft was $115 \pm 90\ \mu m$. There was no increased proliferation of infectious cells (lymphocytes, granulocytes and macrophages) close to the newly formed intima and media. However, there was a proliferation of fibroblasts. No specific infectious cells could be found. Isolated particles of cellulose, which cannot be metabolised by the human body, were detected — mostly located in the new media region (Fig. 3). These were surrounded by few lymphocytes. SEM also revealed a homogeneous endothelialisation inside the grafts with an almost smooth transition to the artery (Fig. 4). The applied Elastica van Gieson staining demonstrated the presence of collagen structure and also proved the absence of an elastic matrix.

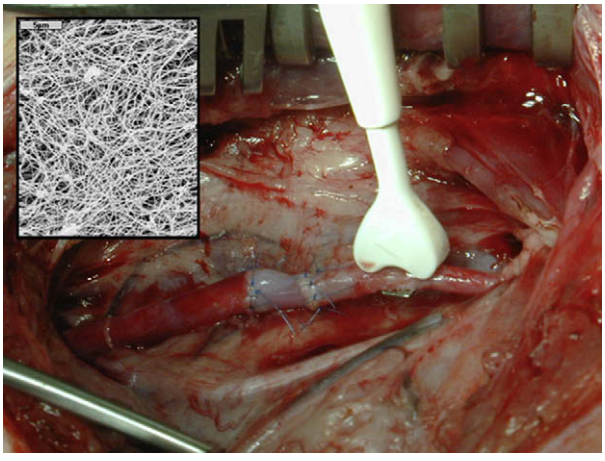

Figure 2 The BC graft implanted in the porcine carotid artery (7/0 Prolene). Patency was subsequently controlled by flow measurement. The ultra-fine network structure of cellulose in the tube (inset). The matrix did not contain cells and required no pre-clotting.

Discussion

There is a need to develop novel vascular grafts that function well in small-diameter applications because small-calibre vascular grafts are associated with high rates of graft failure. Synthetic small-calibre grafts will require patency equivalent to that of autologous arterial vessels. Bioengineering innovations have enabled the seeding of endothelial and smooth muscle cells onto synthetic grafts.[10] Although a seeded graft worked well experimentally, graft preparation is time consuming and technically difficult. If a synthetic graft has good endothelialisation without any seeding, it would be more practical to use it rather than a seeded graft. Further, complete incorporation into host tissues and the maintenance of a viable and self-renewing endothelial layer are the fundamental goals to be achieved when developing a TEBV. Sourcing of cells and modulating their interaction with the extracellular matrix and supporting scaffold have been the focus of intense research. Although the use of TEBV in humans has been limited so far, advances in our knowledge of stem cell precursors and the development of new biomaterials should enable this technology to reach routine clinical practice within a decade. The biocompatibility of a scaffold for tissue-engineered constructs is essential for the outcome. BC consists of completely pure cellulose nanofibrils synthesised by *A. xylinum*. The morphology of single nanofibrils, the network structures of BC and the stress—strain response show similarities to that of a collagen network.[11] BC has high mechanical strength and can be shaped into three-dimensional structures. Cellulose-based materials induce negligible foreign-body and inflammatory responses and are considered biocompatible.[12] The in vivo biocompatibility of BC has never been evaluated systematically.

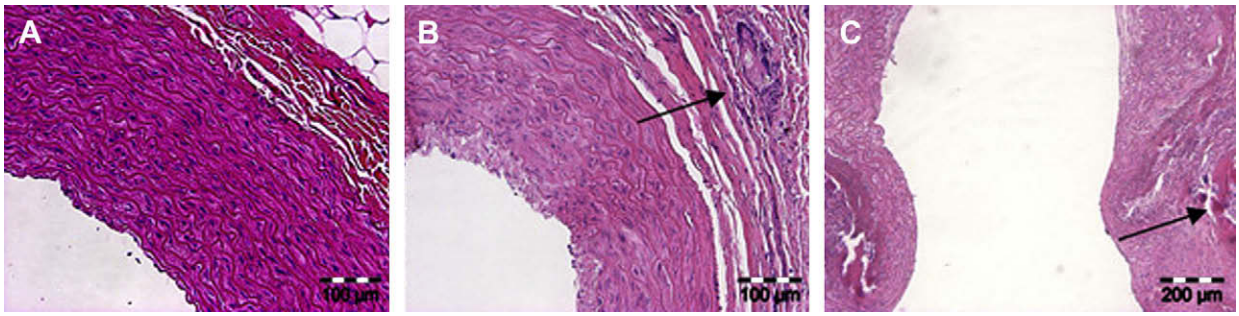

Figure 3 Graft histology (H&E staining) at the experimental end point after 3 months. (a) An untreated segment of the carotid artery; (b) the BC graft after 3 months representing a 'vessel-like' wall structure (arrows) showing a mild infiltration of lymphocytes; (c) cellulose fragments (arrows) are still detectable, which was not out of the ordinary as cellulose is non-degradable in the human organism.

Thus, in the development of tissue-engineered constructs with a BC scaffold, it is necessary to evaluate the in vivo biocompatibility. In the present study, short BC grafts were implanted in the carotid artery of pigs for 12 weeks. The tubes demonstrated an acceptable patency rate (87.5%) with no signs of thrombosis and showed good in situ tissue regeneration for up to 3 months without excessive neointimal hyperplasia or aneurysm formation. Histologically, BC grafts had a three-layered structure, which was reminiscent of the natural arterial structure. The luminal wall of the newly formed tissue (particularly the intima) showed complete endothelialisation with a confluent endothelial layer. There were no macroscopic signs of inflammation around the implants. There were only mild microscopic signs of inflammation around the non-degradable cellulose particles. No fibrotic capsule or giant cells were present. Fibroblasts infiltrated BC, which was well integrated into the host tissue. However, there was a proliferation of fibroblasts, which could be considered a chronic form of infection, although no specific infectious cells could be found. Instead of collagen, the absence of elastic fibres was observed in the neo-tissue. Further biomechanical examination has to be undertaken to demonstrate whether the pliability of the regenerated BC graft can increase to the same level as that of the native artery.

In this study, the precise mechanisms behind in situ regeneration have not been evaluated. In our opinion, there could be two possible reasons for this regeneration. First, the graft macrostructure itself supplies a scaffold for endothelialisation, and cellular infiltration may be originating from the circulating blood or, second, the graft becomes completely incorporated with the host tissue by the infiltration of cells from both anastomotic sites. If endothelial cells inside the graft come only from the anastomotic sites, the BC graft may have a limited critical graft length that reduces its clinical availability. For the next step, we consider the production of longer grafts (up to 10 cm) for use in the grafting procedures.

The degradation of BC has not been fully evaluated in either an in vitro or an in vivo setting. Other cellulose-based materials have, however, shown limited degradation.[13] The idea of a completely degradable material for TEBV is very attractive. In reality, however, problems with degradable materials are clearly present, but are often neglected. Widely used polymers, such as polyglycolic acid (PGA) and polylactic acid (PLA), yield degradation products that, both chemically and mechanically, inhibit cell proliferation in vitro and induce inflammatory reactions in vivo.[14] However, so far, there is no completely biodegradable small-diameter graft suitable for use in the arterial circulation in humans. The transportation of waste products from resorbed polymers is an important concern. The degradation of the material by the cells would be desirable, but it is difficult to optimise and synchronise the degradation time and mechanical properties of polymers. Keeping these difficulties associated with the use of the degradable

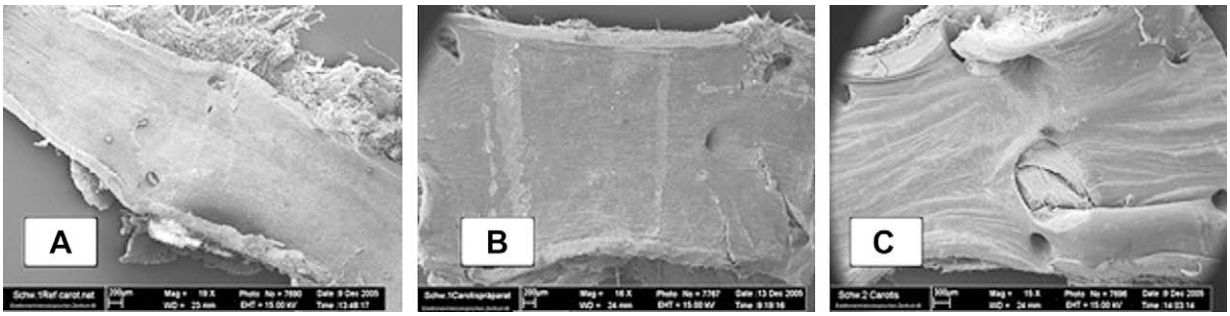

Figure 4 SEM (magnification 16—19×) revealed a good endothelialisation of the BC grafts (b and c). (a) An untreated segment of the carotid artery.

scaffold in mind, a non-degradable, biocompatible scaffold that supports cell adhesion, proliferation and migration would be a major therapeutic advance.

Limitations of the study

This study is a very preliminary evaluation of a novel, bio-engineered, synthetic, small-calibre BC graft. Since grafts were examined for histology alone, no biomechanical properties or hydrodynamics studies at several different time points were performed. The graft compliance and tensile strength will likely continue to change as the graft becomes completely incorporated with the host tissue. Once the process of graft remodelling is completed, the conduit could reach a stable condition in which its mechanical properties will become equivalent to that of the normal tissue over time. There was histological evidence that the macrostructure (trapped water) was degrading during 12 weeks, so we conclude that the microstructure is primarily responsible for the fluctuations in the biomechanical stability of the graft over time.[13] A long-term study is currently being conducted to evaluate changes in the biomechanical stability of the graft.

Conclusion

This preliminary work demonstrates that it will be possible to rapidly fabricate a polymeric tubular graft in vitro and to implant it as a substitute for a small-diameter artery. BC promotes in situ vascular tissue regeneration and does not require pre-treatment in the form of cell seeding. Thus, BC exhibits properties that are promising for its further use in TEBV programmes.

Conflict of interest/funding: none.

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