Bacterial nanocellulose as a new patch material for closure of ventricular septal defects in a pig model

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Abstract

OBJECTIVES: Current materials for closure of cardiac defects such as ventricular septal defects (VSDs) are associated with compliance mismatch and a chronic inflammatory response. Bacterial nanocellulose (BNC) is a non-degradable biomaterial with promising properties such as high mechanical strength, favourable elasticity and a negligible inflammatory reaction. The aim of this study was the evaluation of a BNC patch for VSD closure and the investigation of its in vivo biocompatibility in a chronic pig model.

METHODS: Young’s modulus and tensile strength of BNC patches were determined before and after blood exposure. Muscular VSDs were created and closed with a BNC patch on the beating heart in an in vivo pig model. Hearts were explanted after 7, 30 or 90 days. Macropathology, histology and immunohistochemistry were performed.

RESULTS: Young’s modulus and tensile strength of the BNC patch decreased after blood contact from $6.3 \pm 1.9$ to $3.86 \pm 2.2$ MPa ($P < 0.01$) and $0.33 \pm 0.06$ to $0.26 \pm 0.06$ MPa ($P < 0.01$), respectively, indicating the development of higher elasticity. Muscular VSDs were closed with a BNC patch without residual shunting. After 90 days, a mild chronic inflammatory reaction was present. Moreover, there was reduced muscular tissue overgrowth in comparison with polyester. Proceeding cellular organization characterized by fibromuscular cells, production of extracellular matrix, neoangiogenesis and complete neoendothelialization were found. There were no signs of thrombogenicity.

CONCLUSIONS: BNC patches can close VSDs with good mid-term results and its biocompatibility can be considered as satisfactory. Its elasticity increases in the presence of blood, which might be advantageous. Therefore, it has potential to be used as an alternative patch material in congenital heart disease.

Keywords: Ventricular septal defects • Patch material • Biomaterial • Biocompatibility

INTRODUCTION

Congenital heart defects (CHDs) are the most common birth defects in children and are the most common cause for death in infants under 1 year of age. Ventricular septal defects (VSDs) are among the most common CHDs, accounting for $\approx 20$–29% of all congenital heart malformations [1]. Until now, these defects are usually closed with a patch made out of knitted polyester, expanded polytetrafluoroethylene (PTFE), or autologous or bovine pericardium [2]. However, long-term results have been compromised by material-related limitations. Especially in young children, the amount of patch material with respect to the heart size is large for closure of VSDs or intracardiac baffling. Currently, available materials have limitations with respect to elasticity and compliance which may impair diastolic compliance of the heart. Very recently, Matsuhisa et al. [3] showed that large patch areas were highly correlated with impaired global ventricular function in children with multiple VSDs.

For these reasons, there is a need for a patch material that combines biocompatibility and acceptable tensile strength with elasticity similar to cardiac tissue.

Bacterial nanocellulose (BNC) is a biomaterial with promising properties. The network of cellulose nanofibrils has similarities to...
the collagen network and thereby creates a biomaterial with high mechanical strength, favourable elasticity and a water content of up to 99% [4]. All these characteristics may be favourable for a patch material for closure of VSDs. Moreover, various studies have shown excellent biocompatibility of BNC with minimal local inflammatory reaction [5, 6].

Thus, the aim of this study was the evaluation of a BNC patch for closure of muscular VSDs. Besides feasibility, we were especially interested in the biocompatibility of the material in the presence of blood and characterization of the healing process after VSD closure.

MATERIALS AND METHODS

Fabrication of bacterial nanocellulose patches

BNC was fabricated in the form of patches (Fig. 1A) by using *Gluconacetobacter xylinus* bacteria. Twenty volume parts of Hestrin–Schramm medium were inoculated with 1 volume part of a 7-day-old liquid preculture [7]. A six-well plate was filled with this culture (2.5 ml/well) which was then cultivated at 28°C under static conditions for 14 days. The BNC fleeces were taken from the culture medium, washed with distilled water, treated with boiling 0.1 N aqueous sodium hydroxide for 10 min and washed with distilled water to a neutral reaction of the rinsing agent. The fabricated never-dried fleeces had a diameter of 35 mm and a thickness of 1.4 mm. Figure 1B shows an electron micrograph of the nanofibre structure of these fleeces resembling the structure of a natural collagen network. With the aim of an increase in mechanical stability, some of the product-specific water of an original produced never-dried fleece was removed from the BNC hydrogel. With application of mechanical pressure using water-absorbing materials and additional heating, the thickness of the original BNC fleece was reduced to a 0.7-mm thin BNC patch.

Mechanical testing of bacterial nanocellulose patches

The BNC patches were cut in rectangles with a size of 5 cm × 3 cm and divided into two groups (n = 15). The patches of Group 1 were put into saline, whereas those of Group 2 were put into heparinized fresh pig blood. Before mechanical testing, the thickness of the probes was determined. Tensile tests were conducted using a tensile testing machine (Z010, Zwick, Ulm Germany). After application of a preload of 0.5 N, a strain rate of 10 mm/min was started. All samples were elongated to failure. The tensile strength and Young’s modulus as a measure of elasticity were determined.

Animal model of ventricular septal defect creation and closure with a bacterial nanocellulose patch

**Animals.** German landrace pigs (Oberschleissheim, Germany; 20–30 kg) were used in an experimental protocol that had been
approved by the local Governmental Commission on the Care and Use of Animals (n = 25). Animals received care in compliance with the Guide for the Care and Use of Laboratory Animals. All pig experiments were conducted at the Institute for Surgical Research at the University of Munich.

Anaesthesia and surgical preparation. Anaesthesia and surgical preparation were performed as described before [8]. Briefly, a left lateral thoracotomy in the fifth or sixth intercostal space was performed and the heart was exposed. The entire procedure was carried out without cardiopulmonary bypass. Two purse-string sutures were applied on the left ventricular wall for instrument insertion.

Haemodynamic measurements and echocardiography. Heart rate, arterial pressure and central venous pressure were measured and recorded periodically throughout the initial and follow-up procedures. Additionally, pulmonary artery and pulmonary capillary wedge pressures were obtained at baseline before thoracotomy, after shunt creation, defect closure and at the follow-up examinations.

Surgical procedure and follow-up studies. Creation of muscular VSDs was achieved under echocardiographic guidance as described previously [8]. The diameter of the VSDs was measured in all cases. The patch delivery device and the custom-designed stapler (Fig. 1D) for fixation of the patch have been described previously [8–10]. For the purpose of our study, we substituted the polyester patch with a BNC patch as shown in Fig. 1C.

Closure of the muscular VSDs was performed as described before [11]. The animals did not receive anticoagulant or anti-platelet therapy. In an acute study, the general feasibility of muscular VSD closure was evaluated (n = 5). For mid-term evaluation of the material, three experimental groups with different survival times were formed: 7 (n = 8), 30 (n = 6) or 90 days (n = 6). At the end of the defined survival period, animals were anaesthetized. Haemodynamic and echocardiographic measurements were recorded as described above. Finally, animals were sacrificed by intracardiac injection of 40 mval potassium chloride and hearts were explanted.

Pathology

Tissue preparation. After explantation, hearts were examined macroscopically. The tissue block containing the patch and the anchors were dissected with some surrounding tissue. After briefly flushing with saline, specimens were fixed in formalin (buffered 4%).

Embedding, sectioning, histology and immunohistochemical staining. Techniques for embedding, sectioning, histology and immunohistochemical staining were applied as described previously [12]. Endothelial cells were identified with antibodies against von Willebrand factor (vWF). Antibodies against smooth muscle actin (SMA), vimentin and Movat Pentachrom staining were used for the analysis of cellular and extracellular components of the newly formed tissue. For detailed characterization of inflammatory cells, antibodies against CD3 and chloroacetate esterase (CAE) stain were used.

The macroscopic and histological results were compared with the findings of our previous study on the use of a polyester patch for VSD closure [9].

Statistical analysis

Values are presented as mean ± standard deviation. The paired t-test was used to examine statistical difference. Differences were considered significant if a P-value of ≤0.05 was obtained.

RESULTS

Mechanical testing of the bacterial nanocellulose patch

Young's modulus and tensile strength of the BNC patch decreased significantly after blood contact from 6.3 ± 1.9 to 3.86 ± 2.2 MPa (P < 0.01) and 0.33 ± 0.06 to 0.26 ± 0.06 MPa (P < 0.01), respectively, indicating an increase in elasticity. The decrease in tensile strength was negligible.

Acute feasibility study

The BNC patch was sutured to the thin nitinol wires of the custom-made patch delivery system, which could be easily performed (Fig. 1C) without the development of any cracks or fissures. Furthermore, insertion of the patch delivery system into the sheath and subsequent deployment did not result in any damage to the patch, indicating that the BNC patch can withstand the forces associated with insertion and deployment. Muscular VSDs were successfully created in five pigs (Fig. 2A and B). The mean size of the muscular VSDs was 6.9 ± 1.8 mm. Figure 2C shows positioning of the patch delivery device in front of the defect. Successful closure of the defects as shown in Fig. 2D was achieved in all animals. The animals were haemodynamically stable at all times. Finally, macroscopic inspection showed correct placement of the patches over the defects. The BNC patches revealed no cracks or fissures, indicating sufficient mechanical strength for this procedure (Fig. 3A).

Mid-term evaluation of the bacterial nanocellulose patch

Mid-term results and complications are presented in Table 1. All pigs were haemodynamically stable throughout the procedures. Initially developed insufficiencies of the tricuspid, mitral and aortic valves and changes in haemodynamic values normalized during the follow-up period. There was no evidence of dislocation of the patch or superficial thrombus formation in any of the animals. In addition, there was no decrease of the global left ventricular function in the course of the study.

Histopathology and immunohistochemistry after 7 days of implantation

Macroscopically, yellow-reddish, friable material could be detected on the surface of the BNC patch (Fig. 3B). Histopathological
Figure 2: Representative echocardiographic images throughout an experiment. (A and B) Prominent left-to-right shunting after creation of a VSD. (C) Positioning of the patch delivery system in front of the VSD (marked with arrows). (D) Immediate closure of the VSD after placement of the BNC patch without residual shunting. VSD: ventricular septal defect; BNC: bacterial nanocellulose; LV: left ventricle; RV: right ventricle.

Figure 3: Macropathology of BNC and polyester at different time points. (A) BNC patch immediately after implantation. There are no cracks and the patch is well positioned over the VSD. The BNC patch after 7 days (B), 30 days (C) and 90 days (D) of implantation. (B) Yellow-reddish material on the surface of the patch. (C) The BNC patch is still visible and is covered by a shiny, surface glistening layer. (D) The BNC patch is not visible anymore and covered by newly formed tissue and a shiny, surface glistening layer. (E) Polyester patch after 90 days of implantation [9]. More pronounced tissue overgrowth on top of the patch in comparison with the BNC patch. BNC: bacterial nanocellulose; VSD: ventricular septal defect.
work-up showed that this material consisted of fibrin deposits and incorporated blood cells (Fig. 4A). In addition, longitudinal oriented cells were seen which stained positive for vimentin (Fig. 4D and Table 2). There were dense infiltrations of neutrophil granulocytes and a few T-lymphocytes (Fig. 4A and C, and Table 2) locally related to the patch material. Richardson staining revealed only minimal inflammatory reactions around the nitinol anchors (Fig. 4B).

### Table 1: Mid-term evaluation of BNC patches for VSD closure

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Survival time</th>
<th>7 days</th>
<th>28 days</th>
<th>90 days</th>
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</thead>
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<tr>
<td>Number of animals</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td></td>
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<tr>
<td>Location of the VSD</td>
<td>Mid-muscular (n = 7)</td>
<td>Mid-muscular (n = 5)</td>
<td>Mid-muscular (n = 6)</td>
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<tr>
<td>Size of the VSD (cm)</td>
<td>7.5 ± 1.5</td>
<td>9.3 ± 1.9</td>
<td>9.0 ± 1.5</td>
<td></td>
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<tr>
<td>Success rate (%)</td>
<td>87.5</td>
<td>100</td>
<td>100</td>
<td></td>
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<tr>
<td>Residual shunting</td>
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<td>None (n = 5)</td>
<td>None (n = 6)</td>
<td></td>
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<tr>
<td>Cardiac function at sacrifice (shortening fraction in %)</td>
<td>41.0 ± 5.8</td>
<td>43.3 ± 12.2</td>
<td>39.7 ± 15.1</td>
<td></td>
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<tr>
<td>Survival</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Complications</td>
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<td>Inflammation (n = 1)</td>
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<tr>
<td></td>
<td>Death after 3 days (n = 1)</td>
<td>Death after 3 days (n = 1)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Patch closure failed (n = 1)</td>
<td>Patch closure failed (n = 1)</td>
<td>None</td>
<td></td>
</tr>
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</table>

BNC: bacterial nanocellulose; VSD: ventricular septal defect.

**Figure 4:** Histopathology and immunohistochemistry after 7 days of patch implantation. (A) H&E standard staining: fibrin, erythrocytes and longitudinally oriented cells surrounding the patch as well as dense granulocytic infiltrations. (B) Richardson staining. Few inflammatory cells locally related to the nitinol anchors (N: nitinol anchors; P: BNC patch). (C) CAE staining demonstrating neutrophil granulocytes adjacent to the patch. (D) Superficial, longitudinally oriented cells staining positive for vimentin (P: BNC patch; N: nitinol anchors). CAE: chloroacetate esterase.

Histopathology and immunohistochemistry after 30 and 90 days of implantation

At the time of explantation of the tissue specimen, the patches were well incorporated in the surrounding tissue. On haptic evaluation, the patches appeared flexible and firmly connected within the myocardial tissue. Macroscopically, there was a shiny,
A glistening layer of tissue on the surface of the BNC patches (Fig. 3C) after 30 days of implantation. After 90 days, a small amount of whitish, dull tissue underneath a complete surface glistening layer (Fig. 3D) could be detected. We compared our results with findings of a previous conducted study, where a polyester patch was used for VSD closure [9]. In contrast to the polyester patch, the amount of whitish, dull tissue on top of the BNC patch was less pronounced (Fig. 3E).

Histopathology revealed neoendothelization (Fig. 5B) and a beginning of cellular organization (Fig. 5A). Cells were oriented in an irregular order close to the patch and in a more longitudinal order in the superficial section of the patch and stained positive for vimentin and SMA (Fig. 5D and E). By means of Movat Pentachrom staining, extracellular matrix (ECM) components, mainly proteoglycans, and beginning of neoangiogenesis could be demonstrated (Fig. 5C). After 90 days, the cellular organization and thickness of the newly formed tissue had advanced further (Fig. 6 and Table 2).

Compared with specimen with an implantation time of 7 days, the number of T-lymphocytes was slightly increased. Macrophages and foreign body giant cells were seen in the superficial section of the patch. The number of neutrophil granulocytes adjacent to the patch was reduced. After 90 days, T-lymphocytes close to the patch as well as macrophages and foreign body giant cells in the superficial part of the patch were present. Neutrophil granulocytes could not be detected anymore (Fig. 6G and H).

### DISCUSSION

Many cardiac defects require complex reconstruction of the heart and associated pulmonary and systemic connections. Several different materials (e.g., pericardium, polyester or PTFE) are available for closing cardiac shunt lesions such as VSDs or for reconstruction of outflow tracts or great vessels. However, currently available materials are associated with limitations. Polyester has a high elastic modulus \( \approx 3000 \text{ MPa} \) and is therefore very stiff. Complications such as thromboembolism, infection, shrinkage, calcification or massive tissue overgrowth have been described for synthetic materials [13, 14]. Biological materials such as autologous pericardium have a more favourable elasticity. It is, however, difficult to handle because its edges tend to curl up [15] and it is very thin with a low tensile strength. For this reason, pericardium requires pretreatment with glutaraldehyde, which on the other hand reduces elasticity [16]. In addition, residual shunting due to the lack of coaptation, shrinkage,
Aneurysm formation and calcification has been reported [14]. Further drawbacks of these materials include the inability to grow with the patient’s heart, loss of mechanical strength over time, and limited ability to remodel [2].

Recently, there have been efforts to design tissue-engineered patches [14]. Different cell sources [17-19] and scaffolds have been explored so far, but the optimal tissue-engineered patch has not been found yet. The lack of infective complications is a big advantage of these patches, but drawbacks are still a lack of mechanical strength [14]. Thus, its use is limited to atrial or right-sided heart defects. Furthermore, there are concerns about rapid degradation which can lead to aneurysmal formation [2, 14].

Figure 6: Histopathology and immunohistochemistry after 90 days of patch implantation. (A and B) H&E and Richardson staining: proceeding cellular organization with fibromuscular cells, proteoglycan-rich ECM, and neoangiogenesis as well as neoendothelization (P: BNC patch). (C and D) Vimentin and SMA staining identifying fibromuscular cells (P: BNC patch). (E) Movat Pentachrom identifying ECM components (mainly proteoglycans) within the newly formed tissue (P: BNC patch). (F) Complete neoendothelization confirmed by vWF staining. (G) Mild chronic inflammatory response with foreign body giant cells (arrow) (P: BNC patch). (H) CD3 staining revealing few T lymphocytes around the patch (P: BNC patch). ECM: extracellular matrix; vWF: von Willebrand factor; SMA: smooth muscle actin.
Thus, there is still a significant need for the evaluation of new patch materials.

Materials with elasticity comparable to cardiac tissue and improved biocompatibility allowing for early neoendothelization and resulting in reduced inflammatory response are desired. Until now, studies on the evaluation of the use of alternative patch materials in large animal models are scarce [15, 20].

BNC is a biomaterial that could fulfill many of the above-described requirements. It is a hydrogel composed of a nanofibre network with fibre diameters in the range of 20–100 nm enclosing up to 99% water [4]. In addition, BNC structurally resembles collagen assembly which potentially provides a better scaffold for tissue ingrowth and endothelialization. Many studies have shown the biosafety of this material [4].

In our study, the elasticity of the BNC patch increased after blood exposure which might be favourable for its use as a patch material. Moreover, its elastic modulus is more similar to that of the heart (~0.2 MPa) than polyester (~3000 MPa) or pericardium (~100 MPa). On the other hand, tensile strength decreased in the presence of blood.

Increased elasticity of BNC allowed ameliorated surgical handling: Application of the patch into the delivery system, delivery via catheters into the heart and attachment to the septum with nitinol anchors were achieved without any problems, indicating an acceptable tensile strength. Immediate complete closure of the VSDs could be achieved, which also demonstrates its good compliance. Owing to its improved elasticity, BNC can thereby better adapt to the in vivo demands of a contracting myocardial structure such as the intraventricular septum. VSDs remained completely closed during an observation period of 90 days. At this time point, reduced tissue overgrowth was found in comparison with our previously reported experience with polyester patches [9]. However, further studies are needed to prove if the concept of elasticity is really beneficial. BNC patches could become too elastic and this can negatively impact cardiac function. Therefore, the evolution of elasticity of the BNC patch should be determined in a long-term study.

Biocompatibility studies after implantation of septal defect occlusion devices showed initial formation of thrombotic material in between metal wires and around polyester fibres [21]. In the further course, the thrombotic material was transformed into connective tissue consisting of mainly ECM, embedded fibroblast-like cells and newly formed vessels. In our study, there was a similar behaviour with a typical pattern of thrombus formation with fibrin septation and inclusion of blood cells 7 days after implantation. In addition, we found longitudinally oriented cells which stained positive for vimentin, indicating early accumulation of fibroblasts. These fibroblasts are known to migrate into wounds and are involved in the organization of thrombotic material to form new tissue [22].

At the 90-day follow-up, cellular, extracellular and vascular organization had progressed comparable to histopathological results reported on other patch materials [9, 15]. Cellular infiltration and ingrowth into the patch were only scarcely observed. As previously described, cellular ingrowth might separate nanofibres of the BNC patch potentially affecting the compliance of the material [23]. In addition, tissue ingrowth might be responsible for the shrinkage and change of mechanical properties as seen with synthetic and biological materials [14].

However, better analysis of long-term use and modification of the porosity of the BNC material are needed to determine the impact of cellular ingrowth. Partial neoendothelization was observed after 30 days which was completed after 90 days of implantation in all animals. Staining with vWF demonstrated functional properties of the endothelium as well. These results are comparable to findings on neoendothelization of septal defect occlusion devices [24].

Early and complete neoendothelization is of major clinical relevance because superficial thrombus formation can lead to the development of vegetations or emboli and subsequent organ damage. There are reports on incomplete endothelization of polyester patches with subsequent superficial thrombus formation [25]. In addition, the use of anticoagulant or anti-platelet therapy with all its side effects could potentially be avoided since in our study animals did not receive such a therapy. There was no local thrombus formation in any of the animals, which argues for a low thrombogenicity of the material.

A mild to moderate inflammatory reaction but persistent foreign body reaction had been noted locally related to the polyester fabric of septal closure devices [21]. Our study revealed a mild chronic inflammatory response to the BNC patches. This reaction was comparable to the reaction seen with synthetic patch materials [2]. The inflammatory reaction around the nitinol anchors was minor.

CONCLUSIONS

This is the first study that evaluates BNC patches as a patch material for intracardiac defects. Results show the feasibility of closure of VSDs with BNC patches and excellent surgical handling properties of the material. Immediate and midterm closure was seen in all cases. There were no signs of thrombogenicity. The biocompatibility of BNC in the presence of blood was good and tissue reactions were comparable to currently used materials. Typical stages of a healing response with complete neoendothelization, progression with time of cellular organization and mild chronic inflammatory reactions were seen. With its favourable mechanical properties and its high elasticity, BNC can be considered as an alternative patch material in congenital heart disease.

However, further studies are needed for the evaluation of long-term effects of BNC in vivo such as calcifications, thrombus formation and the general tissue reaction over longer implantation periods, the concept of elasticity and for testing of different modifications of this new patch material such as biodegradable forms of BNC in comparison with other patch materials.

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