Investigation of decellularized biological grafts for human vascular replacement

PhD Thesis

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1. Introduction

One of the most dreadful complications of prosthetic bypass surgery is graft infection. The treatment of septic grafts is professionally considered among the most difficult challenges in vascular surgery, including a high morbidity and mortality rate. In milder cases, only superficial wound infection occurs. However, deep spreading of the inflammation, including the area of the reconstruction, or development of generalized sepsis is a challenging complication for the specialists. These infections can develop also in the postoperative period (early infections) and much later (late infections), too.

There are different methods to treat graft infections, ranging from graft conservative methods to complete graft removal, however, in most cases, survival of the limb and/or the patient can be assured only with successful graft replacement. To effectively achieve this, impregnated (with antibiotics or silver) prosthetic graft or biological graft (which is more advantageous to eliminate infection) can be used.

The advantage of impregnated grafts is their unlimited availability, however, these expensive grafts are subjects to frequent occurrences of reinfections.

The use of autologous grafts, which currently proves to be the best solution, significantly increases the operative risk and their availability is limited.

The application of cadaver, cryopreserved grafts is a favorable option, nevertheless, these grafts are considerably expensive, their storage requires special infrastructure and their availability is, at present, limited.

The third group of biological grafts are xenografts, originating from another species. The advantages of these grafts are the adequate biomechanical properties, unlimited availability and high resistance against infections. The most distinct disadvantage of xenografts is the triggering of the immune response. To reduce immunogenicity, decellularization of the graft is necessary. Decellularization removes all cellular components from the tissue, preserving the original structure of the connective tissue scaffold. Following implantation, host cells migrate into, proliferate and differentiate in the implanted graft's scaffold, and, consequently, the "harmful" immune response will not develop against the new tissue.

Applying xenografts in humans showed promising initial results during the 80's, however, frequent, late complications, related to degeneration of these grafts, generally supplanted it from additional utilization. Nevertheless, the rapid development of vascular surgery in the present century, in addition to the more frequent complications due to the

significantly increased number of graft implantations, focused the attention again to the development of biological vascular grafts.

2. Aims

The goal of our investigation was the development of a biological vascular graft, which combines the favorable properties of the different types of grafts, thus could be an alternative choice for graft replacement in the septic vascular surgical field.

In the **first series** of our experiments, comparative examination of different decellularization techniques was performed, based on light- and electron microscopy findings, as well as mechanical tests. The cell removal effectivity of the different decellularization methods, and the change of the connective tissue fibers in the treated aortic wall were analyzed by histological staining. Resistance against intraluminal pressure of the decellularized grafts and the anastomosis between the treated graft and the native aorta were mechanically tested. Based upon these findings our ideal decellularization procedure was selected for the animal study.

In the **second part** of our investigation, decellularized aortic segments were implanted in the infrarenal part of porcine aorta. In the first part of the animal studies decellularized porcine allograft, then in the second part cell free ovine aorta was utilized. After a 6 month follow-up period, the porcine infrarenal aorta was explanted including the implanted segment, and light- and electron microscopy evaluations were performed. The in vivo patency and macroscopical graft degeneration rate of the implanted grafts were assessed. The reappearance of novel cells and the preservation of connective tissue fibers were evaluated in the implanted segments. We tried to determine the human applicability of these biological grafts in the light of all these examinations.

3. Comparison of the efficiency of decellularization methods based on mechanical tests, light and electron microscopy

3.1. Introduction

Organ or tissue transplantation has shown rapid development in recent decades. As a result of the continuously rising demand, that human resources can no longer entirely supply, the attention focused on the artificial tissue and organ production. The goal of tissue-engineering is the production of decellularized biological material that serves as a scaffold for recipient cells colonization, proliferation, differentiation, and tissue formation. During decellularization procedures, all cellular and nuclear components of the tissue or organ are removed, while preserving the mechanical integrity of the remaining extracellular matrix (ECM). The most important limiting factor in both allo- and xenotransplantation is the anti-donor antibody production by the surface antigens of the donor cells, leading to an inflammatory reaction and immunologic response, and ultimately to an immune-mediated rejection of the implant. This is what the decellularization of the implant may prevent.

Successful utilization of biological scaffolds originates from decellularized organs and tissues (such as tendons, ligaments, nerves, muscles, urine bladder, liver, heart valves, vessels) has also been reported in animal studies, and in human clinical applications. Throughout published literature in regards to this topic, several methods were found to achieve complete decellularization, however, there are several differencies among the methodologies, including mechanical properties, cell removal effectiveness and structure-preserving ability. Thus, effective, widespread materials and techniques were assessed to select the ideal procedure for subsequent implantation, in consideration and with respect to mechanical evaluation and histological examinations.

3.2. Materials and methods

3.2.1. Surgical preparation

Yorkshire pigs, between 22 and 25 kg of weight, were entered into this study. Porcines were anesthetized using continuous intravenous administration of 3 mg/kg ketamine hydrochloride and 1-2 mg/kg xylazine hydrochloride per hour. An intratracheal intubation was

performed, and the pigs were mechanically ventilated with oxygen (1l/min) and N_2O (3l/min) supplemented room air. Slow, intravenous infusion of normal saline maintained hydration throughout the surgery. Total median laparotomy was performed, and the infrarenal part of the abdominal aorta was dissected in the retroperitoneal plane. After 2500 IU of Na-heparine intravenous administration, DeBakey clamps were used for aortic exclusion, while the required aortic sections were removed. The abdominal wall was closed by the use of a loop, and at the final phase of the procedure, a continuous Donati skin suture was performed.

3.2.2. Decellularization methods

Infrarenal aortic segments were freshly excised from porcines. Aortic segments (2cm in length and diameter about 8mm) were next washed in sterile phosphate buffered saline (PBS) to remove residual traces of blood. Eight samples (n=8) were evaluated for each corresponding protocol.

3.2.2.1. Protocol 1

Freshly excised aortic segments were snap frozen to -80°C, and stored at this temperature, awaiting additional, future investigations.

3.2.2.2. Protocol 2

Aortic segments were decellularized through continuous shaking in 1% sodium-duodecyl-sulfate, and 0.05% sodium-azide (NaN_3), in phosphate buffered saline (PBS), at room temperature, for 48 hours. The solution was exchanged every 6 hours. At the end of the decellularization protocol, the aortic segments were washed using PBS for 12 hours to effectively remove residual detergents and cell debris.

3.2.2.3. Protocol 3

Aortic segments were decellularized with 0.075% sodium duodecyl sulfate (SDS) in PBS. Samples were constantly agitated in this solution for 15 hours, at 37°C, before five, 15-minute rinse cycles in PBS, at 37°C.

3.2.2.4. Protocol 4

Aortic segments were decellularized with 0.25% Triton X-100 and 0.25% sodium-deoxycholate in PBS. Samples were in constant agitation in this solution, for 24 hours, at 37°C, prior to a 72-hour wash cycle in Medium 199 with Earle's salts, maintained to 4°C. Following the wash cycle, the marked samples were treated with 100 g/mL RNase A and 150 IU/mL DNase I with 50 mmol $MgCl_2$ in PBS for 24 hours, at 37°C. Following nuclease digestion, samples were washed again using Earle's Medium 199, for 24 hours, at 4°C.

3.2.3. Light microscopy evaluation

Prepared segments of aortic wall were fixed in 10% neutral buffered formalin. Cross-sectional and longitudinal tissue blocks were embedded using paraffin, and 2-4 micrometer thick sections were sliced. Sections were stained applying 3 different methods. Hematoxylin-eosin (HE) staining demonstrated the cell-removal effectiveness of the decellularization protocol. Orcein and Masson-trichrome (MT) histochemistry stains illustrated the structure and preservation of the connective tissue fibers.

3.2.4. Electron microscopy evaluation

Samples of vessels were prepared for transmission electron microscopy (TEM) examination by fixation in 2% formaldehyde and 2.5% glutaraldehyde containing solution. Following fixation, 50-60nm thick sections were cut, and an examination was performed using a JEOL JEM-1200EX II transmission electron microscope, at 80kV. Efficacy of treatments, based on lack or presence of cells, and, the occurrent changes in the ECM structure, were analyzed.

3.2.5. Mechanical tests

Mechanical tests were performed using sections of vessels of 3-4 cm in length. The distal end of the vessels were closed with a continuous suture by using 6/0 Prolen. Side branches were ligated with 2/0 Polypropylene thread. After cannulation of the proximal end of the aorta with an ordinary vessel cannula, this proximal end was fixed and ligated to the cannula by using

2/0 Polypropylene thread. An inflation device was used for the pressure tests. Photo documentation was performed.

3.2.5.1. Pressure test

During pressure tests, treated vessel sections were compared with native aortic segments. After preparing and removal of residual air, the samples were slowly inflated to 225 mmHg with 37 °C PBS, using an inflation device, maintaining this pressure five times for one minute.

3.2.5.2. Suture retention test

First of all, an anastomosis was created between the native and treated vessel segments with continuous 6/0 Prolene suture. After that, the distal ends (the decellularized segment in each case) were closed with a continuous 6/0 Prolene suture. Following cannulation and fixation of the proximal end (the native segment in each case), deaerated samples were slowly inflated to 225 mmHg with 37 °C PBS, maintaining this pressure five times for one minute.

3.3. Results

3.3.1. Light microscopy evaluation

First, the samples underwent Hematoxylin and Eosin (HE) staining to prove, whether blue-stained nuclei disappeared, or not, following the process. Protocol 1 and 3 did not effectively decellularize the aortic tissue, as nuclei appeared much the same as in the native tissue. Protocol 2 and 4 were efficient methods, in which histological sections showed complete removal of cells.

As a result, only at these two successful protocols (Protocol 2 and 4) were both Orcein and Masson-trichrome staining performed. The wall-thickness decreased in each treated groups' samples, however, significant tissue destruction was not notable. The runoff the fibers was decisively regular and arranged in both groups. Nevertheless, only in Protocol 4 could we observe some more undulatory and slightly fragmented fibers, in proximity to the luminal surface, which is generally associated with mild damage in the scaffold.

3.3.2. Electron microscopy evaluation

There was no marked difference in the review of the 2nd and 4th Protocol TEM images. The lack of cellular elements and the retained connective tissue fibers and microstructure were, however, noticeable. Analysis of Protocol 1 and 3 samples were omitted.

3.3.3. Mechanical tests

During the five cycles of inflation there was no rupture of aortic wall or sign of leakage in any cases of the samples, treated by each corresponding protocol. Moreover, both type of sutures (i.e., localized either at the end of the treated vessels or in the anastomotic region) adequately withstood the intraluminal pressure, without any sign of leakage in the suture line in each case.

3.4. Discussion

Varying methods are known for decellularization, with a disparate success rate in cell elimination. The decellularization efficiency depends on the type and origin of tissue, as well as, on the specific physical, chemical and enzymatic method used. The most effective decellularization protocols include combinations of physical, chemical, and enzymatic treatments.

Physical methods, as in the case of fast freezing, may prove to be far more effective in cell lysis, however, not efficient enough towards the effective removal of cellular material, just like mechanical agitation, thus these are only recommended to supplement other treatments.

Among *chemical* methods, ionic detergents, such as SDS, Na-azide or non-ionic detergents, for example, Triton X-100, are widely known and frequently used compounds appreciated for their effectiveness in cellular material removal, including maintenance of the ECM structure. The combination of detergents increases the efficacy of protein extraction from tissue, resulting in more effective cell removal.

It is very difficult to achieve complete cell removal by *enzymatic* treatment alone, in addition, it was shown to be far too aggressive in some studies, causing mild to moderate tissue damage. Thus, enzymatic procedures are not used alone, but to complete the decellularization process, to increase the effect.

It is well-known in scientific and medical research fields, that potential and varying combinations of decellularization methodologies are not perfectly successful in the removal of all cellular components. However, diminishing this content as much as possible without significant damage in the scaffold may result in a higher success rate following implantation.

In the current study, a genuine attempt was made towards effectively assessing all relevant, foregoing methods of decellularization, however, it must be claimed, that the physical method alone, and monophasic use of detergent SDS, demonstrated mild cell removal activity. Additionally, the remaining two decellularization protocols proved to be identical with regards to efficacy, including the total elimination of visible cellular material. The enzymatic reagent containing Protocol 4 caused slightly notable damage within the structure of the extracellular matrix. In consideration of the TEM image of the decellularized vessel, the treated tissues proved to be cell free, together with the microarchitecture of collagen fibers are nearly identical when compared with the native aorta.

Mechanical tests, using 225 mmHg intraluminal pressure (which is nearly two times higher, than the physiological systolic pressure), showed adequate mechanical stability and suture retention strength, without rupture or leakage, in all treated samples. Thus, decellularized vessels may be adapted to implantation.

3.5. Conclusion

There was no significant difference in the efficiency of decellularization between the two effective protocols, however, due to the minimal fiber damage observed in the samples of Protocol 4, Protocol 2 was found more efficient. The mechanical tests did not show any difference in the mechanical behavior of the samples treated by either of the two methods. In the light of histological findings and mechanical tests, considering the shorter incubation time and a substantial cost savings, Protocol 2 was selected for further experiments in animal studies.

4. Investigation of decellularized biografts following implantation during 6 month follow-up

4.1. Introduction

The appearence of artificial grafts revolutionized the treatment of vascular diseases, however, due to their increasing application, prosthetic graft infections became more common. To resolve this problem is still a challenging task for specialists. Graft conservating methods certainly have a place in the treatment of septic graft, but in most cases healing can be only achieved by removing the infected graft. At present, the utilization of autologous grafts is the gold standard, but their use increase significantly the procedural time and perioperative morbidity, thus this method not suitable for high-risk patients. In these cases, silver or antibiotic impregnated grafts are generally applied, resulting in a variable success-rate with regards to the potentiality of reinfection. Application of homografts is a recommended option, generally associated with good results and high resistance against infection, however, there is the potential risk of graft degeneration, in addition, the availability is limited. Applying xenografts (donor graft originates from another species) was supplanted from additional utilization for a long time, because of the unfavorable long-term results, late aneurysm formations and high reocclusion rate due to the graft degeneration. Nevertheless, easy handling and high resistance against infections focused again the attention to the development of biological vascular grafts in the septic surgical field. In consideration of vessel allo- and xenotransplantation, decellularization of the donor tissue may prevent or decrease graft versus host reaction, and subsequent graft degeneration.

In our study, we investigated the post-implantation behaviour, patency and structural changes of the decellularized porcine allo- and ovine xenografts, during a 6 month follow-up.

4.2. Materials and methods

4.2.1. Surgical preparation

Following narcosis, a total median laparotomy was performed. 2500 IU of Na-heparine intravenous administration was performed to prevent clot formation. Antibiotical prophylaxis was achieved by 0,5mg Cefazoline intravenously.

4.2.2. Decellurization of grafts

Aortic segments were decellularized through continuous shaking in 1% sodium-duodecyl-sulfate, and 0.05% sodium-azide (NaN_3), in phosphate buffered saline (PBS), at room temperature, for 48 hours. The solution was exchanged every 6 hours. At the end of the decellularization protocol, the aortic segments were washed using PBS for 12 hours to effectively remove residual detergents and cell debris.

4.2.3. Animal studies

The decellularized aortic grafts were prepared from porcine in the first, and from ovine in the second series. In the first series, 10 allotransplantations (porcine-porcine), and in the second series, 4 xenotransplantations (ovine-porcine) were performed.

Following surgical preparation, and clamping of the aorta, a segment, measuring 1,5-2cm long, was removed, and a corresponding segment of decellularized biological aortic allo- or xenograft, including the appropriate diameter and length, was interposed. After tubing, the abdominal wall was closed, femoral pulses were controlled, and all operated animals were placed under strict observation over the ensuing days. Surviving porcines were transported to their original millieu for the next six months. Following this period, resection of the infrarenal part of the abdominal aorta was performed. Explanted segments of aorta, including the original aortic wall, implanted vessel and the anastomotic region, were evaluated by light- and transmission electron microscopy.

4.2.4. Light microscopy evaluation

In both phases of our study, the following method was used. Implanted and surrounding native segments of the aortic wall were fixed in formalin. Cross-sectional and longitudinal sections were stained applying four different methods. Routine HE staining confirmed the presence of novel cells and the remodelling of the endothelial layer in the implanted graft. Orcein staining for visualization of elastin, including Masson-trichrome staining the collagen, were performed. Re-colonization of myofibroblasts and smooth muscle cells were substantiated by smooth cell actin immunostaining.

4.2.5. Electron microscopy evaluation

In both phases of our study, the same procedure was used to detect newly appearing cells in the implants ECM.

4.3. Results

4.3.1. I. series: Investigation of implanted, decellularized porcine aortic allograft

All of the decellularized grafts, originated from porcines, were surgically easy to handle. Suture retention was adequate, there was no bleeding complication or graft reocclusion in the postoperative period. 9 out of 10 porcines survived the follow-up period. One pig succumbed in ileus.

During the second operation, well-incorporated, and suitably pulsating grafts were perceptible without any pathological transformation or perigraft reaction. The implanted grafts demonstrated a proportional increase in diameter and length.

4.3.1.1. Light microscopy evaluation

Cross-sectional and longitudinal tissue sections were examined, and the latter contained the suture line with a continous transition between the native aorta and the implanted segment. The HE staining showed a large population of novel cells within the implant, including a continuous, regular, single cell-line endothelial layer covering the entire luminal surface. SMA staining proved the presence of novel smooth muscle cells within the graft. Masson-trichrome and orcein staining confirmed the preserved structure of collagen and elastic fibers in all layers.

4.3.1.2. Electron microscopy evaluation

TEM demonstrated the reappearance of native arterial wall cells (fibroblasts, myofibroblasts, smooth muscle cells) among regular connective tissue fibers within the biograft.

4.3.2. II. series: Investigation of implanted, decellularized ovine aortic xenograft

Decellularized ovine grafts, 2cm in length, were implanted in the infrarenal part of porcines aorta. All of the grafts were easy to suture. Bleeding complication, wound healing disorder, ileus were not developed postoperatively. One porcine died in lower extremities ischaemia due to early reocclusion.

During the second operation of the survived 3 pigs, well-incorporated, well-pulsating grafts were perceptible without any signs of degeneration. The xenografts demonstrated a proportional increase in diameter and length.

4.3.2.1. Light microscopy evaluation

Cross-sectional and longitudinal tissue sections were prepared from the explanted aortic segments. HE staining demonstrated a large population of blue-stained cell nuclei within the implant, including regular endothelium. SMA staining proved the presence of normal smooth muscle cell groups within the graft. Masson-trichrome and orcein staining confirmed the preserved structure of collagen and elastic fibers.

4.3.2.2. Electron microscopy evaluation

TEM images of the explanted aortic wall demonstrated the reappearance of fibroblasts and smooth muscle cells, proving the invasion of host cells. The structure of collagen fibers were preserved.

4.4. Discussion

Vascular graft research originates from the beginning of the former century. First experiments were performed using homografts, then in the 80's with xenografts, however, the positive early results were followed by less favorable late outcome, due to their degeneration. Alloplastic grafts appeared in the meantime, and became more and more popular due to their unlimited availability and easy handling. Nevertheless, autologous grafts remained the gold standard in cardiovascular surgery, in terms of primary reconstruction, such as redo surgery, particularly with respect to infected areas.

Autologous graft is not available in an estimated 20-40% of the cases. The use of synthetic grafts for distal bypasses demonstrates low patency rate, and, in addition, the application of impregnated alloplastic grafts in septic surgery offers a variable outcome in terms of the risk of reinfection, thus, the attention turned again to the development of biological grafts.

Presently, the utilization of allografts (homografts) plays an important role in septic vascular surgery, specifically, when autograft is not available. Besides this, xenografts have also come into routine cardiovascular surgical application.

The advantages of xenogeneic materials, compared with alloplastic materials, include superior handling, high resistance to infection and reinfection, minimal bleeding tendency at the suture line, together with unlimited availability. Commercially available patches are typically decellularized by gluteraldehyde, and commonly originate from bovine pericardium.

Several publications described successful application of pericardial xenografts in the septic vascular surgery, however, experiences with usage of these grafts in replacement of the infrarenal aorta are limited. These publications demonstrated positive observations regards to bovine pericardial patches or hand-made bovine pericardial grafts.

In our investigation, cell free vascular biografts were applied for implantation. In the first series, allotransplantations of porcine aortic grafts, and in the second series, xenotransplantations of ovine aortic grafts were performed into the infrarenal part of porcine aorta, with excellent mid-term results. The grafts were surgically easy to handle, proved to be mechanically adequate, and there was no significant bleeding in the suture-line during surgery. Perioperative complication developed only in one case, this pig succumbed in ileus. One complication developed due to reocclusion (ovine aortagraft implanted porcine) during the follow-up period. During the second surgeries, well-incorporated, well-pulsating grafts were found in both animal groups, with no evidence of infection or aneurysmatic degeneration. Histological findings were the same in both animal groups: the reappearence of cells in the implanted scaffold, and a regular, single-layer endothelium without neointima formation, moreover, the lack of inflammatory cell invasion were observed. TEM images showed normal fiber structure and newly appeared, mitotically active cells in the implanted scaffold.

Until this time, we have no experiences with long-term application of xenografts. The late outcome would be probably the same considering the human application of porcine arterial xenografts, such as, in the described earlier attempts, include aneurysmatic degeneration and graft thrombosis. However, the aim of their utilization in septic vascular surgery is to abolish the septic focus, to ensure limb salvage and to improve survivability rates of patients, when there is no other option. Following successful eradication of the potential infection and

complete healing, in the event of any complication, the xenograft may electively be replaced with an additional graft.

4.5. Conclusion

In both experimental phases, we were able to demonstrate excellent results, supported by histology. Degeneration of the implants due to graft versus host reaction has not been developed during allo-, and xenotransplantation. In summary, knowing the late complications of its utilization, however, high resistance to infection and reinfection, usage of decellularized xenografts may offer an ”ultimum refugium” in support of graft replacement, in the treatment of patients suffering from septic vascular graft, if autologous graft or eligible homograft is not available.

5. Novel results of the thesis

1. We confirmed, that monophasic decellularization protocols are not sufficient to achieve complete cell removal in the treated tissues, and a combination of different treatment methods is required for complete decellularization.

2. We have confirmed, that enzymatic decellularization techniques can adversely affect the integrity of the ECM fiber structure.

3. We demonstrated that different decellularization procedures do not significantly influence the mechanical properties of treated vascular grafts.

4. Treated grafts (decellularized according to our ideal method) meet the properties expected in the section of aims, so these cell free vascular grafts may be used for in vivo application.

5. We have performed firstly end-to-end transplantation of decellularized porcine allo- and ovine xenografts into the infrarenal part of porcine aorta, with a 6 month follow-up period.

6. Favorable results, obtained from the animal model and from post-explantation examinations, provide the basis, beside further developments, investigations and close follow-up, for later human attempt of biological vascular graft.