Original Article

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Dehydration improves biomechanical strength of bioartificial vascular graft material and allows its long-term storage

Abstract

Introduction: We have recently reported about a novel technique for the generation of bioartificial vascular grafts based on the use of a compacted fibrin matrix. In this study, we evaluated the effects of a dehydration process on the biomechanical properties of compacted fibrin tubes and whether it allows for their long-term storage.

Materials and methods: Fibrin was precipitated from fresh frozen plasma by means of cryoprecipitation and simultaneously with a thrombin solution applied in a high-speed rotating casting mold. Subsequent dehydration of the fibrin tubes (29/38) was performed in dry air with a dilator inside the tube to prevent the collapse of the lumen. Dehydrated fibrin tubes were stored for six (n = 9) and 12 months (n = 10) at room temperature. Comparative analysis was done on initially generated and dehydrated fibrin tubes before and after storage to evaluate the effects of the dehydration process and storage on the biomechanical properties and structure of the tubes.

Results: Thirty-eight fibrin tubes were generated by high-speed rotation-molding from 142 ± 3 mg fibrinogen with an inner diameter of 5.8 ± 0.1 mm and a length of 100 mm. A centrifugal force of nearly $900 \times g$ compacted applied fibrin, while fluid was pressed out of the matrix and drained from the mold via holes resulting in a 16-fold compaction of the fibrin matrix. Dehydration was characterized by shrinkage of the tubes to a diameter of 3.2 ± 0.2 mm, while the length remained at 100 mm equivalent to a further two-fold compaction. The biomechanical strength of the dehydrated fibrin tubes significantly increased to values comparable to that of native ovine carotid arteries and maintained during the first 6 months of storage. After 12 months of storage, only five of 10 tubes were intact, and only one showed maintained biomechanical strength.

Discussion: Compaction of a fibrin matrix in high-speed rotation-moulding and subsequent dehydration enables for the construction of small-caliber fibrin grafts. Over and above, the dehydration process allows their storage and stockpiling as a prerequisite for clinical use.

Keywords: dehydration; fibrin; tissue engineering; vascular graft.

Introduction

The development of a small-caliber artificial vascular graft still remains a major goal of vascular tissue engineering (TE). Although the major advances in vascular surgery over the last decades are closely related to the clinical introduction of synthetic vascular prostheses [Dacron and expanded polytetrafluoroethylene (ePTFE)], that has made vascular grafts available off the shelf "24/7" with constant quality, at present no ideal vascular prosthesis namely for small-caliber bypass grafting is clinically available. While good patency rates are obtained for large-caliber vascular replacement in the aorta and the iliac arteries up to 90% after 5 years, these synthetic materials provoke subacute

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reactions from the recipient's body that limit their use to large-caliber vascular replacement with a diameter >5 mm [1, 2]. These reactions range from immunologic reactions such as foreign body reactions in the surrounding tissue to humoral antibody-mediated reactions and progress of arteriosclerotic lesions due to inferior biomechanical properties (compliance mismatch). Thus, when used as a bypass in the leg, even with heparin coating, PTFE prostheses have a limited patency rate of up to 59.6% [3].

In consequence, autologous vessels such as the great saphenous vein and the radial artery are still routinely used for small-caliber bypass grafting such as below-the-knee and coronary artery bypass grafting (CABG) [2, 4, 5]. However, their use is limited by quantity and occasional poor quality (varicosis or arteriosclerotic lesions) and may further be restricted by use for previous procedures or amputation. It is estimated that about one-third of the patients have no suitable veins and up to 20% have no adequate autologous bypass material at all [6, 7]. On the other side is a growing demand, namely, for small-caliber grafts due to demographic change in our society with an increasing incidence of cardiovascular disorders [8]. From the information provided by the German societies for cardiothoracic and vascular surgery and vascular and endovascular surgery regarding the numbers of CABG procedures and patients with critical limb ischemia, it can be estimated that at least 20,000–30,000 patients do not have an adequate bypass material for a required operation per year in Germany.

Therefore, the availability of an optimized bioartificial vascular graft constitutes a considerable unmet clinical need. Based on the methods of TE in the past 30 years, various approaches have been developed for the construction of bioartificial vascular prostheses. The goal is the development of a fully biocompatible vascular graft with consistent quality, which is fully integrated into the organism after implantation with structural and functional properties similar to a native artery. The basic principle of the classical approach in TE is the seeding of an adequate scaffold (matrix) with autologous cells of the later recipient to ensure that even matrices with limited biocompatibility do not provoke immunological reactions but are subjected to body's own remodeling mechanisms for repair and regeneration [9]. However, in recent years, biocompatibility of the matrix itself has found to be crucial for complete integration of an implant as well as the ability of cells to migrate inside the matrix from the surrounding tissue and to replace it by newly formed matrix proteins. According to the current state of research, this can only be expected from biological matrices.

Particularly, fibrin provides a number of advantageous properties for the construction of a bioartificial vascular graft. It can be separated from blood or plasma in an adequate amount and molded in nearly all sizes and shapes [10]. It supports attachment, proliferation, and three-dimensional arrangement of various cell types and promotes the formation of extracellular matrix proteins and allows a pronounced remodeling *in vivo* [11–14]. However, severe lack of biomechanical stability has limited its use for implants with a high mechanical load, such as vascular grafts. Different techniques have been developed to increase the stability of fibrin constructs. The addition of mostly synthetic polymers either as structural support outside or inside the matrix to improve cross-linking, however, could diminish fibrin's otherwise excellent biocompatibility [15]. A maturation process of seeded fibrin constructs in a bioreactor *in vitro* is characterized by the gradual replacement by newly formed matrix proteins with a significant increase in biomechanical strength. However, this method is expensive and time consuming [10]. Over and above, seeded structures can be stored for only a few days making a stockpiling for constant availability almost impossible, which is an essential prerequisite for clinical use [16].

We have recently reported about a novel technique for the generation of bioartificial vascular grafts, which relies on the compaction of fibrin during its polymerization from fibrinogen by centrifugal forces in a high-speed rotating mold. While excess fluid is pressed out of the matrix, fibrils get closer together and become more branched and cross-linked leading to a significant increase in biomechanical stability [17, 18].

The method has been successfully evaluated in a first and an ongoing second *in vivo* trial. A pronounced remodeling of the grafts implanted in the carotid artery in a sheep model was found, which was characterized by the ingrowth of cells from the surrounding tissue and a conversion of the implant into a "neo-artery" with great structural and functional similarity to the native vessel [17]. Thus, a long-term course similar to a native artery can be expected in clear contrast to today's vascular bypass materials. Fibrin has a high interspecies conservation, and the excellent results of the study were obtained with human fibrin in a sheep model. Thus, vascular grafts could be engineered from allogenic or even xenogenic fibrin independent of the acute need for stockpiling, when they can then be stored for a longer period of time. The storage of biological structures in aqueous solutions such as saline solution (0.9%) is only suitable for short-term storage of a few weeks [19]. Since the principle of the molding technique with a high-speed rotating mold relies in the

compaction of fibrin by the removal of excess fluid, and in preliminary studies, promising results were obtained by the use of drying preservation techniques (freeze-drying, vacuum-drying, data not shown), in this study, we evaluated the effects of a dehydration process of fibrin tubes in dry air on their biomechanical properties and the ability of long-term-storage of dehydrated fibrin tubes.

Materials and methods

Fibrinogen was precipitated from fresh frozen plasma (FFP) obtained from the blood bank of the Hannover Medical School, which were for any reason not applicable for clinical use.

Fibrinogen precipitation

The frozen plasma was thawed at room temperature and aliquoted in 50-mL Falcon tubes (Greiner Bio-One, Austria). After storage for at least 24 h at −20 °C, plasma was thawed at room temperature and centrifuged at 600 *g* for 3 min. The supernatant was decanted and the remaining hydrous pellet completely dissolved at 37 °C and stored at −20 °C until usage. Fibrinogen concentration was determined in the laboratory of the Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation of the Medical School using the method of Clauss in a coagulometric assay (Sysmex CS-5100, Siemens Healthcare, Forchheim, Germany).

Generation of fibrin tubes

For the engineering of fibrin tubes, 10 mL of fibrinogen precipitation was applied simultaneously with 10 mL of a thrombin solution in a custom-made (research factory, Hannover Medical School) high-speed rotating casting mold. The thrombin solution was composed of 600 U thrombin (Baxter, Unterschleissheim, Germany), 1000 KIU aprotinin (Loxo, Dossenheim, Germany), and 300 U factor XIII (Fibrogammin, CSL Behring, Marburg, Germany) in a 40-mmol calcium chloride solution (Sigma-Aldrich, Hamburg, Germany).

The mold consisted of two removable Teflon® half shells, which were placed inside an outer brass tube together with a cuff at both ends. The cuffs had a 4-mm-wide central hole to allow the insertion of an applicator and narrowing the lumen at both ends of the central cavity to prevent fibrin loss during the manufacturing process. The inner diameter of the mold was 7 mm and the length 100 mm. Altogether, 14 drill holes with a diameter of 0.5 mm within the brass tube allowed for drainage of excess fluid out of the mold (see Figure 1A). During the manufacturing process, the holes were covered with a semipermeable membrane (Leukopor® BSN medical, Hamburg, Germany) to prevent fibrin loss via the drain holes. The mold was driven by an electric motor with a rotation velocity of 15,000 rpm. The resulting g force was calculated by the formula: centrifugal force (g) = $1119 \times 10 - 5 \times r \times rpm2$ (r = radius of the mold).

Application of the fibrinogen precipitation and the thrombin solution was done separately via two lancets (0.8 × 120 mm, BBraun, Melsungen, Germany). Both lancets were placed in a third lancet
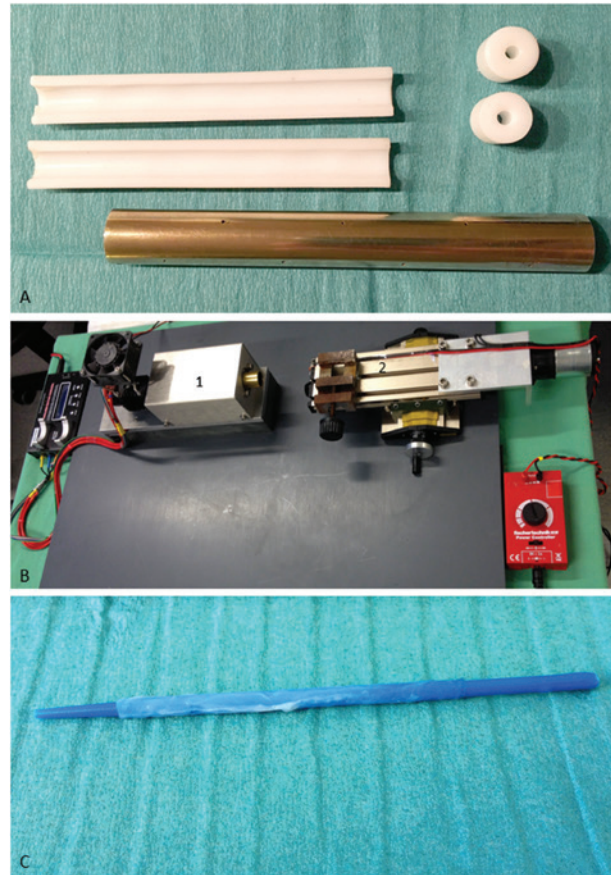

Figure 1: The precipitated fibrin preparation was applied simultaneously with an activating thrombin solution into a high-speed rotating mold (A). The mold consisted of two 100-mm-long removable Teflon-half shells in a brass tube. The diameter of both half shells together was 9 mm. Teflon cuffs at both ends narrowed the lumen to 4 mm. Drill holes with a diameter of 0.5 mm within the brass tube allowed for the drainage of excess fluid out of the mold. The mold was driven by an electric motor (B-1). The applicator consisted of two 120-mm-long lancets in a third wider lancet and was fixed on a movable slide (B-2) and pulled in out of the mold at a defined velocity during the application of the fibrin precipitation. For dehydration, 29 fibrin tubes were thread on the dilator of an 8F-sheath (C) to prevent collapse of the lumen during dehydration process and placed in a Falcon tube with sodium chloride on the bottom to achieve humidity of <30%.

(2 × 100 mm), which was mounted on a mobile slide driven by a separate electric motor allowing for the adjustable movement of the applicator (see Figure 1B). The applicator was moved into the mold and out at a defined speed during the application of both the fibrin precipitation and the thrombin solution. The mold and the lancet were autoclaved prior to use to guarantee sterility.

Dehydration process

Twenty-nine of the generated fibrin tubes were thread on the dilator of 8F-sheaths (Avanti, Cordis, Norderstedt, Germany) to prevent

collapse of the lumen during dehydration process and placed in a Falcon tube (see Figure 1C). The bottom of the Falcon tube was covered with sodium chloride, which was replaced if necessary to achieve humidity of <30%. The process was continued under room temperature without changes in environmental temperature until the fibrin had completely attached to the dilator. Afterwards, the fibrin tubes were each stored in a Falcon tube at room temperature. Structural and biomechanical characterization of the dehydrated fibrin tubes was performed directly after the dehydration process and after 6 months and 12 months of storage. Thus, the generated fibrin tubes can be divided into four groups:

– initial (without dehydration process) (n = 9)
– de-/rehydrated (after dehydration process) (n = 10)
– stored 6 months (stored for 6 months after dehydration process) (n = 9)
– stored 12 months (stored for 12 months after dehydration process) (n = 10)

Before use for structural and biomechanical characterization, each fibrin tube was rehydrated in calcium chloride solution (40 mmol/L) for 1 h at room temperature. Afterward, it was pulled off the sheath and stored in sodium chloride solution (0.9%) overnight to ensure that the rehydration was completed before the further characterization and that there would be no potential further swelling of the matrix, which might decrease the measured biomechanical properties.

Structural and biomechanical characterization

Structural and biomechanical tests were performed on every fibrin tube and carotid arteries (n = 9), which were resected in a previous *in vivo* study (Niedersaechsisches Landesamt fuer Verbraucherschutz und Lebensmittelsicherheit, reference number: 33.9-42502-04-11/0493) for comparative analysis.

Structural analysis

Histologic analysis: Specimens of each fibrin segment were fixed in paraformaldehyde (4%) overnight at 4 °C. Cryosections of each specimen (10 μm) were analyzed on digital photographs of light microscopic views (Axio Observer, Zeiss Jena, Germany) using the digital image processing software Axio vision (version 4.8.2, Zeiss Jena). Wall thickness was measured by means of image processing software (ImageJ, NIH, MD, USA). For estimation of the degree of compaction, the volume of the wall of the fibrin tubes was calculated by the formula: $\pi R(1)^2 * 100 - \pi R(2)^2 * 100$ (R(1) = outer radius of the fibrin tubes, R2 = inner radius of the fibrin tubes).

Electron microscopy: Fibrin tubes before and after the dehydration process were fixed in a solution with 1.5% (v/v) paraformaldehyde (Sigma-Aldrich), 1.5% glutaraldehyde (Merck, Darmstadt, Germany), and 150 mM HEPES (Sigma-Aldrich) at pH 7.35 for 30 min at room temperature. After critical-point-dried (Baltec CPD 030, Bal-Tec, Lichtenstein), specimens were sputtered on the luminal surface with an ultrathin gold layer (Polaron SEM-Coating System). Specimens were observed using Philips SEM-505 (Philips, Netherlands).

Biomechanical characterization

For the characterization of the biomechanical properties of the fibrin tubes, tensile strength and burst pressure were measured. To determine the tensile strength, three specimens (1×1 cm) were taken from each end and the middle of each fibrin tube. The specimens were placed in a mechanical testing device (ATP Messtechnik, Ettenheim, Germany) and pulled apart until structural failure, while length and applied force were recorded. From the measured values, the wall tension was defined (kPa). The secant modulus of elasticity was calculated from the max. tensile strength (kPa)/Δ length (mm) at the point of structural failure, and the modulus of elasticity was calculated by the formula: Δ tensile strength/Δ length (mm) in the most relevant range between a tensile strength of 9 and 17 kPa corresponding to an intraluminal pressure of 70–130 mmHg. For the measurement of the burst strength, a 3-cm-long specimen of each fibrin tube was placed in a custom-made chamber device. The proximal end was connected via a silicon tube to a reservoir filled with saline solution and the distal end to a pressure monitor system. Saline solution was pumped into the fibrin tube with a flow rate of 10 mL/min until it ruptured. Burst strength was defined as the highest pressure recorded before rupture. The integrity of the fibrin tubes after storage was evaluated by the measurement of burst strength. If pressure could be built up during the filling with fluid, the fibrin tubes were defined as intact. Suture strength was measured by pulling out a suture (Prolene 7-0, Ethicon, Norderstedt, Germany), which was placed at one end of the fibrin tubes, respectively, the carotid arteries 1 mm apart from the edge. The strength that was needed to pull out the suture was defined as the suture strength (N).

Statistical analyses

All statistical analyses were performed with GraphPad Prism, Version 6 (GraphPad Software Inc., USA). Mean and standard deviation (SD) were calculated for all continuous variables. The significance of the differences was determined on the basis of Tukey's test for the analysis of variance (ANOVA). Differences were considered significant at p ≤ 0.05.

Figure 2: By high-speed rotation molding, 100-mm-long fibrin tubes were generated with an outer diameter of 7 mm (A). During dehydration, the tubes shrank to the diameter preset of the dilator inside (B). After 6 months of storage in dry air, all fibrin tubes were intact (C), whereas after 12 months, most of the tubes had fissures (indicated in D).

Results

Fibrinogen precipitation

Fibrinogen concentration of the plasma (FFP) was 2.39 ± 0.2 mg/mL. From 100 mL of plasma, 5.1 ± 0.8 mL of fibrinogen preparation was precipitated with a mean fibrinogen concentration of 13.6 ± 4 mg/mL.

Generated fibrin tubes

Thirty-eight fibrin tubes with a length of 100 mm and an outer diameter of 7 mm were generated from 10 mL of fibrin precipitation with a total amount of fibrinogen of 142 ± 3 mg (see Figure 2). There were no significant differences in the amount of the applied fibrinogen between the four groups. The wall thickness of the fibrin tubes before

Table 1: Parameters of the fibrin tubes.

| Parameter				Fibrin tubes
	Initial	De-/rehydrated	Stored 6 months	Stored 12 months
Diameter (mm)	5.8 ± 0.1	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.1
Length (mm)	100	100	100	100
Wall thickness (μm)	614 ± 74	730 ± 150	677 ± 92	672 ± 40
Volume of the wall (mL)	1.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.02
Biomechanical properties				
Tensile strength (kPa)	23.9 ± 9.2	55.9 ± 11.4	63.7 ± 13	33.3 ± 18.2
Burst strength (mmHg)	201 ± 78	576 ± 86	624 ± 108	173 ± 236
Secant modulus (kPa/mm)	2.5 ± 1.3	4.8 ± 1.1	5.9 ± 1.7	3.6 ± 2.3
Elasticity modulus (kPa/mm)	4.4 ± 2.9	6.2 ± 2.4	8.4 ± 4.7	5.2 ± 4.6
Suture strength (N)	1.1 ± 0.5	3 ± 0.5	2.8 ± 0.7	1.6 ± 0.5
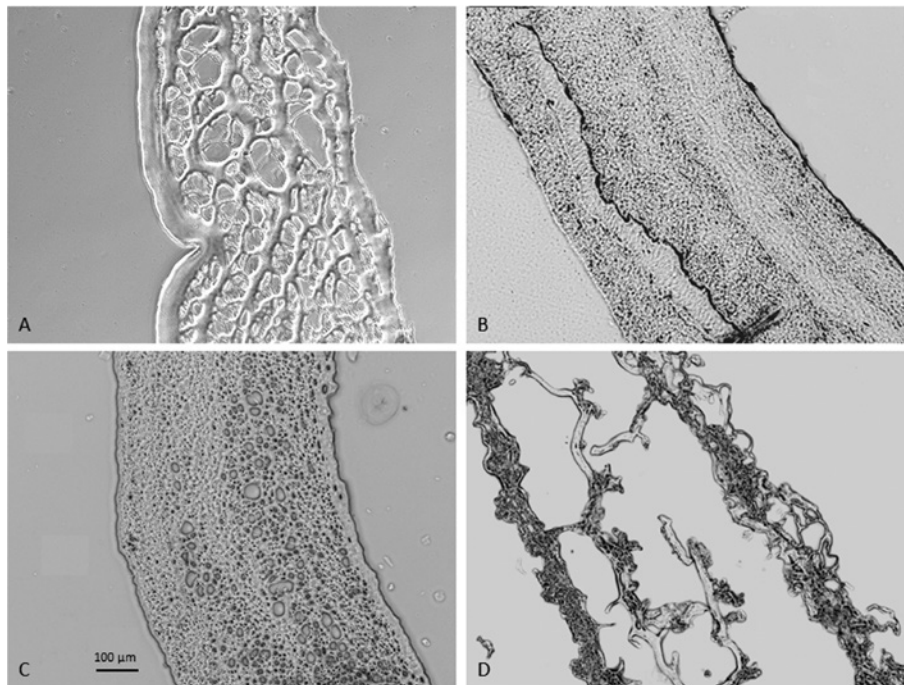
Figure 3: Phase-contrast microscopy of sections of fibrin tubes before dehydration (A), after dehydration before storage (B), stored for 6 months (C) and 12 months (D). Semiquantitative analysis showed that fibrils were denser packed with an increase in branching and linkage of the fibrils after dehydration. The structure maintained during the first 6 months of storage, whereas fibrin matrices stored for 12 months and showed an unstructured composition.

dehydration was 614 ± 74 µm with a resulting inner diameter of 5.8 mm (for details, see Table 1). Calculated from these values, the volume of the wall of the fibrin tubes was 1.2 ± 0.1 mL, which means that approximately 94% of the applied volume (fibrin precipitation + thrombin solution) was pressed out of the matrix and the mold during the molding process corresponding to a 16-fold compaction of the fibrin matrix.

Dehydration process

During dehydration, shrinkage of the fibrin tubes occurred. A dilator inside the tube prevented lumen collapse and kept the length of the attached fibrin tubes at 100 mm. Thus, shrinkage was limited to the diameter given by the size of the dilator inside (2.7 mm). After rehydration, fibrin tubes could easily be withdrawn from the dilator. While the thickness of the wall of the dehydrated tubes increased to 730 ± 150 µm, the inner diameter decreased to 3.2 ± 0.2 mm and the volume to 0.6 ± 0.1 mL corresponding to a further two-fold compaction of the fibrin matrix (see Table 1).

Structural analysis

Semiquantitative analysis of the cryosections revealed that in the de-/rehydrated matrices, in comparison to the matrices before dehydration (initial), the space between the fibrils decreased. The fibrils are more densely packed and appear to be more branched (see Figure 3A and B). Electron microscopic scans of the luminal surface also showed that there is a compaction of the fibrils. This compaction makes the luminal surface appear much smoother than the surface of the non-dehydrated or static-generated fibrin matrix. If the space between the fibrils is referred to as pore size, this decreases significantly during compaction and, again, significantly during the dehydration of the fibrin (see Figure 4).

Biomechanical characterization

The tensile strength of the fibrin tubes before dehydration (initial) was 23.9 ± 9.2 kPa (min. 15.8–max. 45.9), burst strength was 201 ± 78 mmHg (120–325), and suture strength was 1.1 ± 0.5 N. After dehydration, the biomechanical strength was significantly increased to (tensile strength) 55.9 ± 11.4 kPa (42.4–77.3) and (burst strength) 576 ± 86 mmHg (450–675) ($p < 0.05$ each). Thus, the stability reached a level
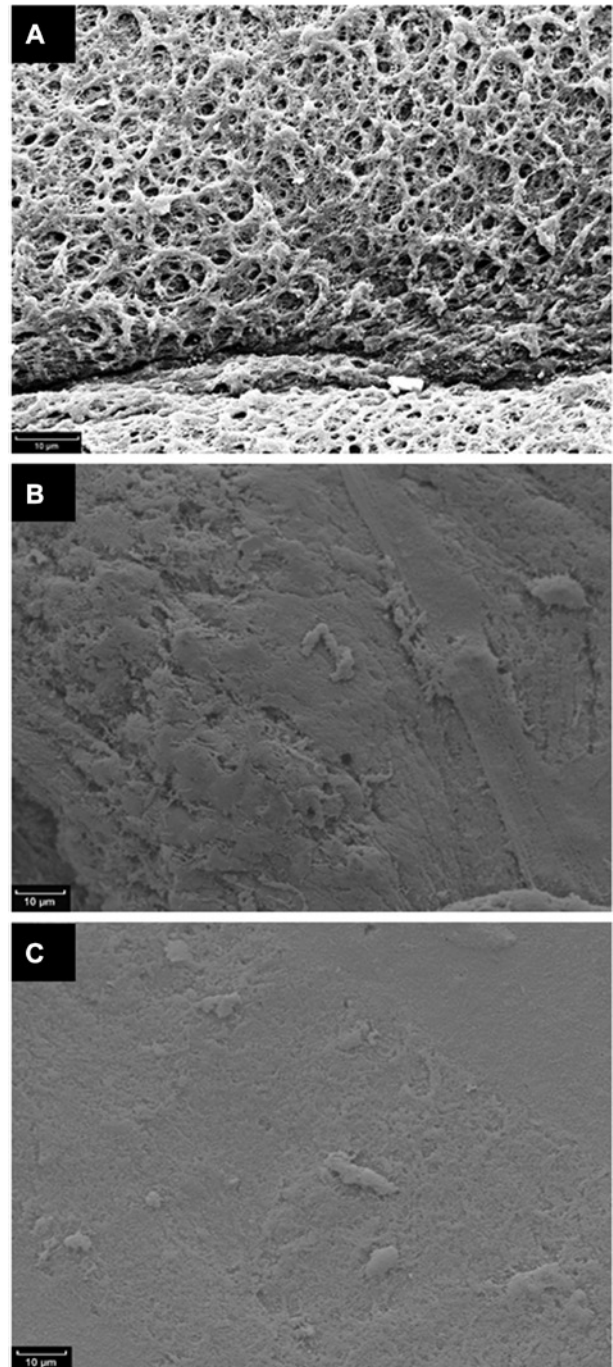

Figure 4: Electron microscopy of the luminal surface of fibrin tubes, which were generated under static conditions (A), generated in the rotation mold before (B) and after de-/rehydration (C) showed a compaction of the fibrils when generated in the rotation mold and a higher compaction when fibrin matrices were dehydrated. This compaction makes the luminal surface appear much smoother than the surface of the non-dehydrated or static-generated fibrin matrix.

of about two thirds of the native carotid artery. The secant elasticity increased from 2.5 ± 1.3 to 4.8 ± 1.1, and the elasticity modulus increased from 4.4 ± 2.9 to 6.2 ± 2.4 kPa/mm
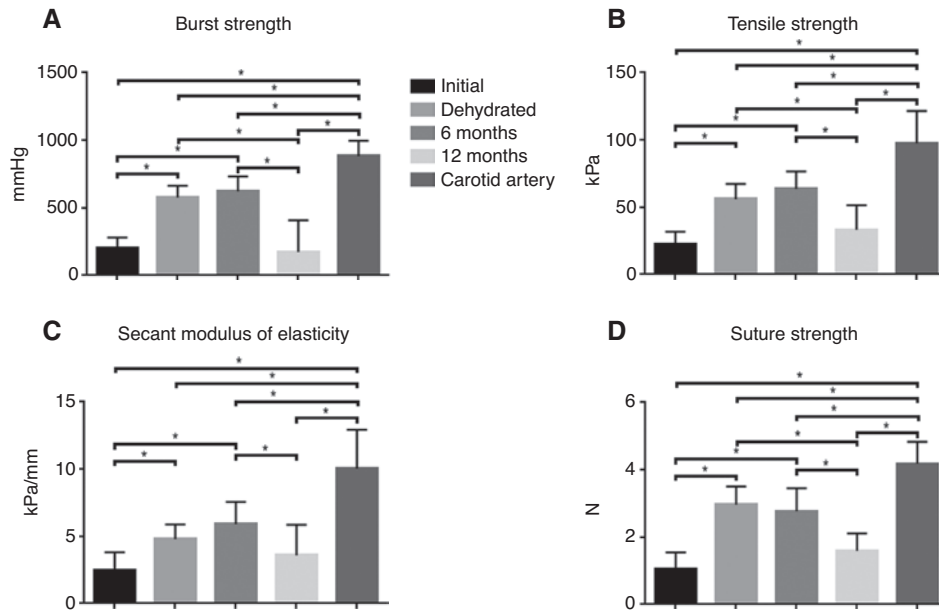
Figure 5: Biomechanical properties [burst strength (A), tensile strength (B), secnt modulus of elasticity (C) and suture strength (D)] were significantly highest of carotid arteries and significantly lowest of the segments, which were stored for 12 months (12 months) in comparison to other groups (p < 0.05).
Dehydration process resulted in a significant increase in biomechanical stability in comparison to the fibrin tubes initially after their generation (initial). Dehydrated fibrin tubes reached approximately two-thirds of the biomechanical strength of the carotid arteries, and biomechanical strength maintained during storage for 6 months (6 months), but significantly decreased after 12 months of storage. All significant differences (p < 0.05) between two groups are marked with bracket*.

(p < 0.05). The suture strength as another important factor for the implantability beside the burst strength increased to 3 ± 0.5 N (for comparison: carotid artery 4.2 ± 0.7 N) (see Figures 5 and 6 and Table 1).

Storage

After 6 months of storage, all fibrin tubes were patent and showed structural integrity (see Figure 2C). The biomechanical properties remained approximately at the same level as the dehydrated, not stored tubes with no significant increase in the tensile strength to 61.7 ± 15.1 (38.4–77.4) kPa (p = 0.06) and burst strength to 624 ± 108 (470–760) mmHg (p = 0.86) (see Figure 5). The elasticity increased to 5.9 ± 1.7 (secant modulus) and 8.4 ± 4.7 (elasticity modulus) (p = 0.94) and the suture strength to 2.5 ± 0.9 N (see Figures 5 and 6). In contrast, after 12 months of storage, less than half of the segments (n = 4/9) were intact. The other five tubes had fissures mostly in longitudinal direction, so that during the measurement of the burst strength, no pressure could be built up (see Figure 2D). Phase-contrast microscopies revealed a loosened structure in comparison to the fibrin structure after dehydration and after storage for 6 months (see Figure 3). Only

one fibrin tube showed excellent biomechanical stability (tensile strength: 71.4 kPa, burst strength: 660 mmHg). All other segments had a significantly decreased biomechanical stability (for details, see Table 1 and Figures 5 and 6).

Discussion

Centrifugal forces in a high-speed rotating mold led to a compaction of the fibrin matrix. As approximately 90% of the fluid was removed, fibrin was compacted by a factor of 16 resulting in a significant increase in biomechanical stability to 200 mmHg, whereas under static conditions, the generated fibrin matrix has a burst strength of only 20–25 mmHg [17]. As the balance between sufficient drainage of excess fluid and loss of fibrin through the wells is the critical point in compacting fibrin with significant enhancement of biomechanical properties, coverage of the drain holes in the mold with a semipermeable membrane was found to be a reliable method to keep this balance. While drainage of excess fluid and, thereby, compaction of the fibrin matrix significantly increases biomechanical strength of the fibrin matrix, this effect can be enhanced by a subsequent dehydration of the fibrin matrix in a simple experimental setup in dry air. Although
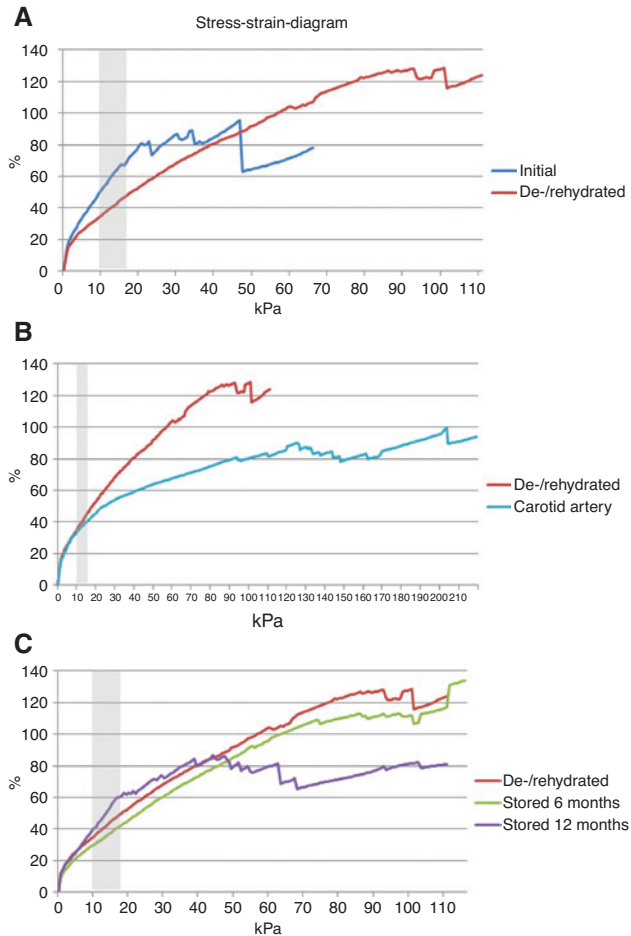
A

B

C

Figure 6: Stress-strain diagram of fibrin tubes before and after dehydration (A), dehydrated fibrin tubes in comparison to native carotid arteries (B), and dehydrated fibrin tubes before and after storage for six resp. 12 months (C). Shown is the elongation in% (Y-axis) compared to the applied tensile force (X-axis). The most relevant range between a tensile strength of 9 and 17 kPa corresponding to an intraluminal pressure of 70–130 mmHg. Decreased elasticity is represented by a plainer curve. Dehydration led to a significant increase in stability and at the same time to a decrease in elasticity (A). The graphs of dehydrated fibrin tubes and ovine carotid arteries showed a similar course in the range between 9 and 17 kPa, while the biomechanical strength of native carotid arteries was significantly higher corresponding with a significant plainer curve progression (B). The graphs of dehydrated fibrin tubes before storage and after 6 months show a very similar course, while elasticity increased after 12 months of storage. However, the curve is masked here by partially good results.

the dehydration technique might be optimized in future experiments, we were able to show that the dehydrated segments can be stored at room temperature for at least 6 months. Storability for at least 6 months allows for a stockpiling, and availability "24/7" is a prerequisite for a potential clinical application.

Histological and electron microscopic studies revealed that the dehydration leads to further densification of the fibrin and decrease in the spaces between the fibrils. It is known that cellular ingrowth and as a prerequisite of remodeling *in vivo* depends on the pore size of the matrix [20]. While fibrin has been proven to be an ideal matrix for the seeding with different cell types and allows their ingrowth and three-dimensional arrangement, further *in vivo* experiments must prove the suitability of the dehydrated fibrin tubes. To what extent the decrease in the space between the fibrils (pore size) has an effect on the adhesion and migration of cells as a decisive step of remodeling cannot currently be estimated. Presumably, only the planned further *in vivo* experiments can answer the question as to whether remodeling is delayed or even impaired and whether the compaction of the surface may even have a positive effect on a decrease in thrombogenicity. A technique for coating the luminal surface with heparin to reduce thrombogenicity has already successfully been established (data not shown) to reduce thrombogenicity of the fibrin graft until complete endothelialization of the graft *in vivo*. However, a decreased cellular ingrowth *in vivo* could also prevent premature degradation of the matrix and subsequent aneurysmal dilatation. If the remodeling proves to be insufficient, a subsequent seeding with the cells of the recipient would be conceivable and has already been established. In this way, generated seeded fibrin tubes are currently being evaluated in a second *in vivo* study.

Storage of the fibrin segments was done in an atmosphere with no defined humidity. A too low humidity might have caused the fibrin segments to became brittle during storage between months 6 and 12. Storage under defined conditions regarding humidity or an altered composition of the atmosphere or even the storage at lower temperatures might allow for a longer storage without loss of structural integrity and functionality than the observed 6 months in this study. Over and above, the sterility of the fibrin tubes after the dehydration process and storage was not proven. In the presented study, the fibrin tubes were generated from sterile compounds in a sterile mold, but contamination of the fibrin tubes might be one reason for the observed loss of stability and integrity during storage. A subsequent sterilization of the fibrin tubes is an important feature for a potential later clinical use because it ensures sterility of the implant regardless of the manufacturing process and is planned to be evaluated in further experiments.

An effect that occurs during drying, which is absent in other preservation methods such as cryopreservation and may not be desired in other approaches is the shrinkage of the fibrin segments. Here, it has found to be a positive side effect as it can be used for the reduction in the diameter of the fibrin tubes to a desired size. This size is

defined by the dilator placed inside the tube. After rehydration, the fibrin tubes keep the reduced diameter, while the length of the tubes remains the same. This enables the generation of fibrin segments with a diameter of 3–3.5 mm and a length of 20 cm (data not shown). Generation of fibrin tubes with such dimensions has turned out to be problematic so far due to vibrations of the applicator, which prevented a uniform distribution of fibrin in the mold. Particularly, the generation of small-diameter vascular grafts with a length of >20 cm is relevant for a potential later clinical use.

At present, no small-diameter tissue-engineered graft has reached the stage of clinical use. It was not possible to combine functionality and full biocompatibility with sufficient biomechanical strength to achieve long-term functionality of a graft. Whereas the attempt to generate a "neo-artery" *in vitro* with functional properties comparable to a native artery has made the procedures more and more complicated, expensive, and time consuming; up to now, it has not been possible to construct a bioartificial vascular graft that permanently exhibits the functionality of a native artery after implantation *in vivo*. With regard to clinical use, less the elaborate *in vitro* generation of a "neo-artery" seems to be promising, but rather, the reduction of the manufacturing process to the construction of a fully biocompatible acellular device with the desired size and shape, which underlies a distinct remodeling process with the conversion to a neo-artery after implantation *in vivo* [21].

This concept is also referred to as *in situ*-TE. Again, also in *in situ*-TE, fibrin has been proven to be an ideal matrix that allows cellular ingrowth from the surrounding tissue and its gradual replacement by extracellular matrix proteins. However, up to now, structural support, e.g. by a guiding polymer was needed, which again could limit remodeling processes by itself or by its degradation products. This problem is also associated with the exclusive use of a biodegradable polymer as guiding structure for the formation of a neo-artery. Particularly, degradation products might trigger immunological reactions in the tissue, which lead to a decreased functionality up to the reversal of the remodeled wall structure [22, 23].

The implantation of decellularized allogeneic or xenogeneic vessels, which are remodeled *in vivo* to a functional artery, has, so far, not shown the expected results, due to a decreased ingrowth of cells and insufficient endothelialization of a highly thrombogenic luminal surface induced by exposed collagen components [24, 25]. In contrast, vascular grafts composed from compacted fibrin can be coated with heparin to diminish thrombogenicity and have a great potential for remodeling *in vivo* into functional vascular structures.

Thus, although potential effects of storage on the functionality of fibrin grafts has to be evaluated in upcoming *in vivo* studies, their use has a high potential capability for successful translation into clinical use. The possibility of storage and stockpiling demonstrated in this study is an important prerequisite for this purpose.

Author Statement

Author Contributions

Thomas Aper: conceptualization; data curation; formal analysis; methodology; project administration; writing – original draft. Mathias Wilhelmi: co-researcher. Ulrike Boer: co-researcher. Skadi Lau: co-researcher. Andres Hilfiker: head of laboratory. Axel Haverich: head of department.

References

[1] Chiu KW, Davies RS, Nightingale PG, Bradbury AW, Adam DJ. Review of direct anatomical open surgical management of atherosclerotic aorto-iliac occlusive disease. Eur J Vasc Endovasc Surg 2010;39:460–71.

[2] Klinkert P, Post PN, Breslau PJ, van Bockel JH. Saphenous vein versus PTFE for above-knee femoropopliteal bypass. A review of the literature. Eur J Vasc Endovasc Surg 2004;27:357–62.

[3] Samson RH, Morales R, Showalter DP, Lepore MR, Jr, Nair DG. Heparin-bonded expanded polytetrafluoroethylene femoropopliteal bypass grafts outperform expanded polytetrafluoroethylene grafts without heparin in a long-term comparison. J Vasc Surg 2016;64:638–47.

[4] Cameron A, Davis KB, Green G, Schaff HV. Coronary bypass surgery with internal-thoracic-artery grafts–effects on survival over a 15-year period. N Engl J Med 1996;334:216–9.

[5] Klinkert P, van Dijk PJ, Breslau PJ. Polytetrafluoroethylene femorotibial bypass grafting: 5-year patency and limb salvage. Ann Vasc Surg 2003;17:486–91.

[6] Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al. Heart disease and stroke statistics–2010 update: a report from the American Heart Association. Circulation 2010;121:e46–215.

[7] Angelini GD, Newby AC. The future of saphenous vein as a coronary artery bypass conduit. Eur Heart J 1989;10:273–80.

[8] Eckstein H-H. Epidemiologie und Versorgung von Gefäßpatienten in Deutschland. In: Debus ESG-FW, editor. Operative und interventionelle Gefäßmedizin. Berlin, Heidelberg: Springer; 2012:163–84.

[9] Niklason LE. Techview: medical technology. Replacement arteries made to order. Science 1999;286:1493–4.

[10] Aper T, Teebken OE, Kruger A, Heisterkamp A, Hilfiker A, Haverich A. Development of implantable autologous

small-calibre vascular grafts from peripheral blood samples. Zentralbl Chir 2013;138:173–9.

[11] Aper T, Teebken OE, Steinhoff G, Haverich A. Use of a fibrin preparation in the engineering of a vascular graft model. Eur J Vasc Endovasc Surg 2004;28:296–302.

[12] Lau S, Eicke D, Carvalho Oliveira M, Wiegmann B, Schrimpf C, Haverich A, et al. Low immunogenic endothelial cells maintain morphological and functional properties required for vascular tissue engineering. Tissue Eng Part A 2018;24:432–47.

[13] Lau S, Schrimpf C, Klingenberg M, Helfritz F, Aper T, Haverich A, et al. Evaluation of autologous tissue sources for the isolation of endothelial cells and adipose tissue-derived mesenchymal stem cells to pre-vascularize tissue-engineered vascular grafts. BioNanoMaterials 2015;16:309–21.

[14] Long JL, Tranquillo RT. Elastic fiber production in cardiovascular tissue-equivalents. Matrix Biol 2003;22:339–50.

[15] Tschoeke B, Flanagan TC, Cornelissen A, Koch S, Roehl A, Sriharwoko M, et al. Development of a composite degradable/ nondegradable tissue-engineered vascular graft. Artif Organs 2008;32:800–9.

[16] Sarkar S, Schmitz-Rixen T, Hamilton G, Seifalian AM. Achieving the ideal properties for vascular bypass grafts using a tissue engineered approach: a review. Med Biol Eng Comput 2007;45:327–36.

[17] Aper T, Wilhelmi M, Gebhardt C, Hoeffler K, Benecke N, Hilfiker A, et al. Novel method for the generation of tissue-engineered vascular grafts based on a highly compacted fibrin matrix. Acta Biomater 2016;29:21–32.

[18] Aper TW, Hoeffler H-K, Benecke N, Haverich A. Engineering of small diameter tubular compacted fibrin matrices. BioNanoMaterials 2016;17:13–7.

[19] Drury PJ, Olsen EG, Ross DN. Morphological assessment of sucrose preservation for porcine heart valves. Thorax 1982;37:466–71.

[20] Murphy CM, Haugh MG, O'Brien FJ. The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering. Biomaterials 2010;31:461–6.

[21] Cleary MA, Geiger E, Grady C, Best C, Naito Y, Breuer C. vascular tissue engineering: the next generation. Trends Mol Med 2012;18:394–404.

[22] Best CA, Fukunishi T, Drews J, Khosravi R, Hor K, Mahler N, et al. Oversized biodegradable arterial grafts promote enhanced neointimal tissue formation. Tissue Eng Part A 2018.

[23] Goh ET, Wong E, Farhatnia Y, Tan A, Seifalian AM. Accelerating in situ endothelialisation of cardiovascular bypass grafts. Int J Mol Sci 2014;16:597–627.

[24] Aper T, Haverich A, Teebken O. New developments in tissue engineering of vascular prosthetic grafts. Vasa 2009;38:99–122.

[25] Boer U, Spengler C, Jonigk D, Klingenberg M, Schrimpf C, Lützner S, et al. Coating decellularized equine carotid arteries with CCN1 improves cellular repopulation, local biocompatibility, and immune response in sheep. Tissue Eng Part A 2013;19:1829–42.

Reviewer Assessment

Thomas Aper*, Mathias Wilhelmi, Ulrike Boer, Skadi Lau, Nils Benecke, Andres Hilfiker
and Axel Haverich

Dehydration improves biomechanical strength of bioartificial vascular graft material and allows its long-term storage

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Reviewers' Comments to Original Submission

Reviewer 1: Balazs Gasz

May 21, 2018

Reviewer Recommendation Term:	Accept with Minor Revision
Overall Reviewer Manuscript Rating:	70
Custom Review Questions	**Response**
Is the subject area appropriate for you?	4
Does the title clearly reflect the paper's content?	4
Does the abstract clearly reflect the paper's content?	4
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	3
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	5 - High/Yes
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	4
Please rate the accuracy of methods.	3
Please rate the statistical evaluation and quality control.	5 - High/Yes
Please rate the appropriateness of the figures and tables.	3
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

In the present paper authors evaluate the dehydration and storageof fibrin tubes applied as vascular small diameter graft. During the study the effect of dehydration process and long-term storage has been evaluated. In previous studies the team described the method of fibrin vascular graft preparation and its function in experimental model, with very promising results. According to the results and conclusion of the study it can be summarized that the method presented by authors enable construction, dehydration and storages of small diameter vascular grafts for 6 months, whereas after 12 mounts of storage period the majority of grafts lost their integrity (5 of 10 was intact according to the observation methods).

The study has crucial importance and - after clarifying some further questions - the method has potential application as a product of clinical use in yet unsolved area. The investigations are well designed, the statistical analysis and the presentation of the results are correct.

The most important deficiency of the paper is the simplified methodology for analysis of vascular graft tubes. Several, more impressive and comprehensive analysis of the tube-structure, like micro CT, electron microscopy, permeability or calorimetry may increase the impact of the study and also may help to understand the processes occurring during storage and dehydration method. On the other hand, in the present form, the methods demonstrated in the article are eligible for conclusions and performing the first evaluations.

It can be the trouble with downloading, but tables cannot be reach in the manuscript. The completion of manuscript with tables is essential for understanding.

Minor concerns and questions:

* The introduction section is far too long and descriptive. Reduction of introduction section is suggested for being more focused to the main topic of the paper. Focusing on problem of long-term storage of vessel tubes and short review of the structural changes during the dehydration and storage may help to better understanding for and interest of the readers.

* Although the wall thickness was thoroughly measured in the groups, was the efficacy and reproducibility of dehydration process assessed by measuring the amount of water-loss? Have any study, including any preliminary study performed to measure the weight change of tubes during the dehydration and rehydration? And how was it changed during shorter and longer-term storage?

* It was described, that the devices where sterile during the procedure. How can the vascular tubes sterilized and was there any further assessment to clarify the sterility of the grafts?

* On figure 3 it is described and visible, that the tubes after 12 months can lost integrity and fissures occur. How was the integrity of the tubes after 6 months evaluated, in detail? Can any permeability analysis assess the smaller than visible fissures or morphological changes? Is any data available about the leakage/permeability of the tubes in the 4 group?

* Discussion section is suggested to be completed with one-two sentences about comparison with further TE procedures and decellularized models and biomechanical properties.

In conclusion the paper describes novelties with potentially high clinical impact, after the completion of these minor deficiencies the paper might be able for publication.

Reviewer 2: Dirk Wilhelm

May 16, 2018

Reviewer Recommendation Term:	Revise with Major Modification
Overall Reviewer Manuscript Rating:	55

Custom Review Questions	**Response**
Is the subject area appropriate for you?	2
Does the title clearly reflect the paper's content?	3
Does the abstract clearly reflect the paper's content?	3
Do the keywords clearly reflect the paper's content?	3
Does the introduction present the problem clearly?	5 - High/Yes
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	3
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	4
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	1 - Low/No
Does the reader get new insights from the article?	3
Please rate the practical significance.	4
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	2

Please rate the appropriateness of the figures and tables.	3
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	3
Please judge the overall scientific quality of the manuscript.	3
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

Dear authors, thank you for submitting your manuscript upon dehydration of vascular grafts to the ISS. The topic herein is of relevance and the problem discribed well adressed. Also the applied methodology and analysis are well performed, however some issues of the article require revision. First of all, the introduction is much too long and not to the point, as more focused to the overall problem of vascular grafts than on the topic of dehydration and storage of bio implants. The same attributes to the discussion, that is adressing the results of the study only in part and that omits relevant aspects. E.g. how do you explain the gain in strength after dehydration/rehydration that remains over a period of 6 months and is then lost? Why is the wall thickness of the dehydrated (and rehydrated?) graft thicker than before this process. Do you expect the graft being integrated and remodeled in the same matter as in a non-dehydrated implant? Is the concept of rehydration overnight practicable in clinical routine and necessary at all? Please revise the discussion accordingly. Some other questions also arised during reviewing that also require revision. Most importantly, please specify the dehydration process more in detail, or was it performed really in such manner? Was there a change of temperature or reduction of the humidity of air, as typical in cryoconservation? Statistical analysis do not include comparative analysis and are only descriptive. Furthermore, a table that visualizes all results in comparison would be of highest interest. Finally, there are some linguistic and spelling errors among the text that need to be corrected. The topic presented in the article is relevant and the methods applied appropriate, however the article is unfocused and includes some unrelevant information while omitting interesting aspects on the other hand. Accordingly, I would recommend thorough revision of the article.

Authors' Response to Reviewer Comments

Jun 06, 2018

Dear Reviewers,

Thank you for reviewing our manuscript. We have revised the manuscript according to your comments. Namely the sections introduction and discussion were completely revised and shortened. Changes in the manuscript are underlined.

Introduction

Reviewer 1

The introduction section is far too long and descriptive. Reduction of introduction section is suggested for being more focused to the main topic of the paper. Focusing on problem of long-term storage of vessel tubes and short review of the structural changes during the dehydration and storage may help to better understanding for and interest of the readers.

Reviewer 2

First of all, the introduction is much too long and not to the point, as more focused to the overall problem of vascular grafts than on the topic of dehydration and storage of bio implants.

- We have completely rewritten the introduction section and shortened it by one third. Figure 1 has been removed.

Materials and methods

Reviewer 1

It was described, that the devices where sterile during the procedure. How can the vascular tubes sterilized and was there any further assessment to clarify the sterility of the grafts?

- A subsequent sterilization of the fibrin tubes is an important feature for a potential later clinical use and is planned to be evaluated in further experiments. In the presented study fibrin tubes were generated from sterile compounds in a sterile mould. But, sterility of the fibrin tubes after dehydration process and storage was not proven. Contamination of the fibrin tubes might one reason for the observed loss of stability and integrity during storage. We have added this point to the discussion section.

Reviewer 2

Most importantly, please specify the dehydration process more in detail, or was it performed really in such manner? Was there a change of temperature or reduction of the humidity of air, as typical in cryoconservation?

- The dehydration process was done as described under room temperature without changes of the environmental temperature (we have added this point to the description). Changes in the temperature would be needed in a way, if cryoconservation of vacuum drying would have been done. But, in this admittedly simple experimental setup, fibrin tubes were only dried in dry air. To achieve an environment with dry air, humidity was reduced only by addition of sodium chloride to the flask. We have referred to this relatively simple experimental set-up in the manuscript (materials and methods and discussion). There is no question, that this process as well as the further storage can be optimized in further studies. The purpose of this study was to examine the extent to which dehydration of the fibrin matrix affects its properties. And since the well-known methods that allow storage of a biological matrix also work with dehydration, we have evaluated the possibilities of storage of the dehydrated fibrin matrix. This can certainly be optimized. We have added this point in the discussion.

Results

Reviewer 1

The most important deficiency of the paper is the simplified methodology for analysis of vascular graft tubes. Several, more impressive and comprehensive analysis of the tube-structure, like micro CT, electron microscopy, permeability or calorimetry may increase the impact of the study and also may help to understand the processes occurring during storage and dehydration method. On the other hand, in the present form, the methods demonstrated in the article are eligible for conclusions and performing the first evaluations.

- We have added electron microscopic scans to the figures. Unfortunately, we did not make these scans from specimens of all groups for comparative studies. MRT-studies have so far not brought the desired information due to an insufficient at present. We are still working on this issue.

Reviewer 1

Although the wall thickness was thoroughly measured in the groups, was the efficacy and reproducibility of dehydration process assessed by measuring the amount of water-loss? Have any study, including any preliminary study performed to measure the weight change of tubes during the dehydration and rehydration? And how was it changed during shorter and longer-term storage?

- We did not weigh the fibrin tubes. This is in our opinion a defective method, because some water adheres to the segments in the state immediately after their generation namely in the lumen. We have chosen the calculation of the volume of the cured fibrin/ the volume of the wall of the segments itself, for estimation of water less/ the degree of compaction.

On figure 3 it is described and visible, that the tubes after 12 months can lost integrity and fissures occur. How was the integrity of the tubes after 6 months evaluated, in detail? Can any permeability analysis assess the smaller than visible fissures or morphological changes? Is any data available about the leakage/permeability of the tubes in the 4 group?

- Integrity of the fibrin tubes after storage was evaluated by the measurement of burst strength. If pressure could be built up during the filling with fluid, the fibrin tubes were defined as intact.

In 5 of 9 fibrin tubes, which were stored for 12 months, during measurement of the burst strength no pressure could be built up due to fissures in the tubes.

These points were added to the manuscript.

Reviewer 2

Statistical analysis do not include comparative analysis and are only descriptive. Furthermore, a table that visualizes all results in comparison would be of highest interest.

- We revised the tables to address this point. Significant differences are marked now in figure 5.

Discussion

Reviewer 1

Discussion section is suggested to be completed with one-two sentences about comparison with further TE procedures and decellularized models and biomechanical properties.

- We added this point to the discussion.

Reviewer 2

The same attributes to the discussion, that is adressing the results of the study only in part and that omits relevant aspects. E.g. how do you explain the gain in strength after dehydration/rehydration that remains over a period of 6 months and is then lost?

- We have completed revised the discussion section and added the points mentioned by the reviewers.

Why is the wall thickness of the dehydrated (and rehydrated?) graft thicker than before this process?

- During dehydration process a dilator was placed inside the tubes to prevent lumen collapse, to which the fibrin attached. This attachment kept the length of the fibrin tubes at 100 mm. Thus, shrinkage was limited to the diameter given by the size of the dilator inside (2.7 mm).

While the wall thickness increased after dehydration, the total volume of the cured fibrin in the wall of the segments decreased. We described this in more detail in the manuscript to clarify this point to the.

Do you expect the graft being integrated and remodeled in the same matter as in a non-dehydrated implant?
- While fibrin has been proven to be an ideal matrix for the seeding with different cell types and allows their ingrowth and three-dimensional arrangement, further in vivo experiments must prove the suitability of the dehydrated fibrin tubes. To what extent the decrease of the space between the fibrils (pore size) has an effect on the adhesion and migration of cells as a decisive step of remodelling cannot currently be estimated. Presumably, only the planned further in vivo experiments can answer the question as to whether remodelling is delayed or even impaired and whether the compaction of the surface may even have a positive effect on a decrease in thrombogenicity.

Is the concept of rehydration overnight practicable in clinical routine and necessary at all? Please revise the discussion accordingly.
- A rehydration of the dehydrated fibrin tubes for 1 hour is sufficient for the further use. We kept the fibrin tubes in in sodium chloride solution (0.9%) overnight to ensure that the rehydration was completed before the further characterization and that there would be no potential additional swelling of the matrix for example after a potential implantation in vivo, which might decrease the measured biomechanical properties.

Tables
Reviewer 1
It can be the trouble with downloading, but tables cannot be reach in the manuscript. The completion of manuscript with tables is essential for understanding.
Reviewer 2
A table that visualizes all results in comparison would be of highest interest.
- All tables are summed up in one table now.

Reviewers' Comments to Revision

Reviewer 1: Balazs Gasz

Jun 11, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	75

Custom Review Questions	**Response**
Is the subject area appropriate for you?	4
Does the title clearly reflect the paper's content?	4
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	3
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	4
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	4
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	4
Please rate the accuracy of methods.	3
Please rate the statistical evaluation and quality control.	5 - High/Yes
Please rate the appropriateness of the figures and tables.	4

Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:
According to the changes performed in the manuscript, I suggest the paper for publication in ISS.

Reviewer 2: Dirk Wilhelm

Jun 14, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	75

Custom Review Questions	**Response**
Is the subject area appropriate for you?	3
Does the title clearly reflect the paper's content?	4
Does the abstract clearly reflect the paper's content?	4
Do the keywords clearly reflect the paper's content?	4
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	4
Are units and terminology used correctly?	4
Is the number of cases adequate?	5 - High/Yes
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	3
Does the reader get new insights from the article?	4
Please rate the practical significance.	4
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	4
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	5 - High/Yes
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:
Dear authors, thank you for revising the manuscript according to the reviewers comments. Accordingly, the manuscript has improved in clarity and significance so I would support is publication.