Aus der Klinik für Herzchirurgie der Universitätsklinik Düsseldorf

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Einfluss einer Laminin-Beschichtung auf die autologe in vivo Re-Besiedelung von dezellularisierten Aorten-Implantaten

Dissertation

zur Erlangung des Grades eines Doktors der Medizin

der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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2021

Als Inauguraldissertation gedruckt mit Genehmigung der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

gez.:
Dekan:

Erstgutachter: Prof. Dr. med. Alexander Assmann

Zweitgutachter: Prof. Dr. med. Alexander Rehders

Zusammenfassung (German)

Die koronare Herzkrankheit ist eine der weltweit am häufigsten auftretenden Krankheit, die auch hohe Zahlen von Komplikationen hat. Die Auswahl an Materialien für die Revaskularisation ist nicht unbegrenzt. Tissue Engineering erzielt gute Ergebnisse als alternative Methode zur Verwendung von Gefäßtransplantaten in Prothesen mit kleinem Durchmesser, es gibt jedoch noch einige ungelöste Probleme. Die Dezellularisierung ist eine bekannte Methode zur Verringerung der Immunantwort gegen fremde Organismen, obwohl die Langzeitergebnisse durch dezellularisierte Transplantate nicht verbessert wurden. Um die Biokompatibilität der Transplantate zu verbessern, verwendeten wir Laminin als Beschichtungssubstanz für Gefäßprothesen. Wir wollten die Rezellularisierung beschleunigen und die Degeneration und die Immunantwort der implantierten Prothesen in vivo reduzieren. Daher wurde der Rattenaortenbogen dezellularisiert und mit Laminin beschichtet und in die systemische Blutzirkulation der Wistar Ratten implantiert (n=23). Die Lamininbeschichtung wurde durch ein Fluoreszenzverfahren nachgewiesen, und die Lamininbeschichtung blieb mindestens acht Wochen bestehen. Unbeschichtete Transplantate wurden als Kontrollgruppe verwendet (n=14). Der Fortschritt der Rezellularisierung der Transplantate wurde zwei und acht Wochen nach der Implantation untersucht. Obwohl nach zwei Wochen keine relevante Zellmigration beobachtet wurde, fanden wir heraus, dass Laminin die Rezellularisierung der Intima nach acht Wochen signifikant beschleunigt ($p = 0.0048$) und die Intima-Hyperplasie reduziert ($p = 0.0149$). Die Kalzifizierung der implantierten Transplantate war nicht betroffen. Darüber hinaus waren die untersuchten Entzündungsmarker der implantierten Transplantate negativ. Die untersuchten Transplantate wurden in beiden Gruppen ohne großen Unterschied mit Alpha-Glattmuskel-Actin-positiven Zellen bedeckt. Langzeit-Experimente werden benötigt, um die Haltbarkeit und Degeneration der Transplantate zu beobachten. Laminin in Kombination mit anderen Beschichtungsmitteln sollte auch als selektives Beschichtungsmittel luminal und adventitiell untersucht werden. Zusammenfassend scheint Laminin eine vielversprechende Zukunft bezüglich der Verbesserung der Biokompatibilität von Gefäßimplantaten zu haben.

I. Summary (English)

Coronary heart disease is one of the most frequently occurring complicative illnesses worldwide. The choice of materials for the revascularization is not unlimited. Tissue engineering is achieving good results as an alternative method to generate small diameter vascular prostheses, however there are still some unsolved problems. Decellularization is a known method for reducing the immune answer against foreign materials. To improve the biocompatibility of the grafts, we employed laminin as a coating agent for vascular prostheses. We aimed to accelerate the recellularization and reduce the degeneration and the immune answer in implanted prostheses in vivo. Therefore, rat aorta arch was decellularized and coated with laminin and implanted in the systemic circulation of Wistar rats (n=23). Based on fluorescence binding, laminin coating was proven to persist for at least eight weeks. Uncoated grafts were used as a control group (n=14). The grafts recellularization progress was examined two and eight weeks after implantation. Although after two weeks no relevant cell migration was observed, we found out that laminin significantly accelerates the intima recellularization after eight weeks ($p = 0.0048$) and reduces the intima hyperplasia ($p = 0.0149$). Calcification of the implanted grafts was not affected. Moreover, examined inflammatory markers of the implanted grafts were negative. The examined grafts were covered with alpha-smooth muscle actin-positive cells in both groups without big difference. Long-term experiment time is needed to observe the long-time durability and degeneration of the grafts. Laminin in combination with other coating agents should be also examined as separate coating agent luminally versus adventitially. In summary, laminin may have a promising future to improve the biocompatibility of vascular implants.

II. Abbreviations

CABG	Coronary artery bypass grafting
ECM	Extracellular matrix
IMA	Internal mammary artery
PDMS	Plasma-modified polydimethylsiloxane

III. Contents

1. Introduction

Coronary artery bypass grafting (CABG) was introduced in the 1960s, becoming from this time forward one of the most frequently performed surgical operations on the heart. CABG is performed at a rate of 44 per 100,000 individuals in a year [1]. Since the 1960s, due to techniques improvement of the operation and despite the higher risk-profiles of patients, outcomes have been significantly improved. Moreover, CABG is the preferred therapy for patients with diabetes mellitus and with poor ejection fraction compared to interventional therapy like stenting. Though, the early mortality rate of CABG is 1-2%, patients with higher risks, for example emergency cases or multiple comorbidities, have nevertheless higher mortality. About 3% of patients with early myocardial infarction in the early postoperative phase have clinical hemodynamic instability resulting from early graft failure [1]. The choice of conduits to bypass coronary lesions has been a continuous debate since using of single internal mammary artery (IMA) graft has proved to have superior long-term outcomes over saphenous vein grafts. Though, there are 3 guidelines with recommendations for increasing the use of arterial conduits, the rate of multiple arterial grafting with IMA grafts or the radial artery remains persistently below 10% [1], [2]. The bilateral IMA grafting has a high risk of deep sternal wound infection. The radial artery grafting shows significantly better graft patency than vein grafts, however most patients that undergo CABG operations have atherosclerosis. Despite all knowledge about risk factors and predisposition to atherosclerosis, there is no really established prophylaxis or drugs therapy against atherosclerosis. Nowadays, almost 80% of all CABG grafts are saphenous veins because of being readily available and easy to harvest [1]. The recent studies have shown excellent outcome with vein grafts. The major disadvantage is its tendency for progressive failure during follow-up impairing long-term clinical outcomes [3]. The failure rate in one-year follow-up ranges from 15% to 29%, and up to 40-50% of failed venous grafts at 10-years postoperatively after CABG [2]. To reduce the failure of veins, the harvesting techniques have been improved (no- or less-touch), and intraoperative storage with heparinized saline and autologous whole blood have been used. All these methods have shown reduced long-term complications, but have not prevented intima hyperplasia [2].

Due to the expected long-term failure of autologous grafts in younger patients, the limited availability of autologous grafts, as well as limitations of re-revascularization, tissue engineering offers interesting approaches to invent grafts with improved properties.

An ideal graft should satisfy some expectations, such as better outcome and no failure: no thrombosis, no infection and no calcification. Easy harvesting and not complicated implantation should also be targets.

Tissue engineering uses methods, substances, cells or the combination of them to improve, restore or maintain the cells, tissue function or whole organ [4]. Decellularization is a technique of tissue engineering that has been developed and further improved in the last two decades. The idea of decellularization is to remove ideally all cellular content of a tissue (including deoxyribonucleic acid and ribonucleic acid) while preserving the extracellular matrix (ECM). The decellularization process remains decisive to avoid the immune response but also must preserve the structural components of the ECM, to provide a sufficient scaffold and accelerate sufficient recellularization. Different kinds of decellularization protocols using multiple types of solutions, including detergents or enzymes to remove the cells from prostheses, varying incubation times and concentrations of used solutions have been investigated. Decellularization has shown to minimize the inflammatory reaction by the host organism. Because of removing all donor cell antigens, the immune reaction of the host organism is diminished, which can also be shown in rat implantation models [5], [6]. The 24h trypsin EDTA decellularization has shown good results of saving the ECM structure, however their cell detritus was perceptible, which may serve as a substrate for calcification [7]. Zhou et al. compared 4 types of decellularization and found out that the decellularized porcine leaflets are significantly thrombogenic [8]. For the present study, we have chosen a method of decellularization including the cytoskeleton inhibitor latrunculin, for which we have recently reported improved decellularization results [9].

The small diameter vascular prostheses have durability issues, not at last because of their small lumina and resulting thrombogenic risk. Only a few successful clinical

implantations of tissue-engineered vascular grafts have been reported until now [10].

Nowadays, we have innovative methods of preparing xenoprostheses and artificial protheses with tissue engineering techniques, which must be improved to transfer newly developed grafts to daily hospital routine. Previous studies have already shown the benefit of decellularization to improve the durability of non-autologous grafts [9], [11], [12]. The decellularized prostheses have shown decreased inflammation and calcification, however the speed and quality of cellular repopulation have to be further improved to enhance the biocompatibility of the implants [13], [14].

In our rat model, decellularized implanted grafts with or without coating have shown recellularization of both the intima and the media, whereas bioactive coating has accelerated the luminal cellularization [13]–[16]. Nevertheless, the problem of concurrent intima hyperplasia was not solved till today. Therefore, the search for a coating agent for quick recellularization and inhibition of intima hyperplasia is of a great interest.

A major problem of the implanted grafts is calcification in the long term. Calcification is a central process of graft degeneration, therefore playing a crucial role for bad long-term outcomes. The pre-implantation anti-calcification treatment to avoid fast degeneration should be improved.

Laminin is the glycoprotein of the extracellular matrix, responsible for cell migration, cell attachment and differentiation [17]. Laminin is an appropriate coating agent with the function of promoting cell migration and adhesion of epithelial cells to achieve cell attraction to implanted grafts. These functions are suitable for fast recellularization and consequently remodeling of decellularized grafts.

A suitable animal experiment is necessary to evaluate the *in vivo* recellularization and degeneration of grafts and to compare the graft modification of treated and not treated grafts. For the *in vivo* studies in our experimental research, a well-established rat model is used for the valve conduit and vessels implantation [18].

All experiments were approved by the state animal care committee (reference number 84–02.04. 2012. A391) and conducted following the national animal welfare act.

1.1. Aim of the work

To make one more step towards using tissue-engineered grafts in daily clinic routine, we have examined laminin as coating agent for decellularized prostheses in a small animal implantation model. We aimed to study laminin as a potential coating agent to minimize intima hyperplasia, while allowing for sufficient recellularization. The recellularization rate is important regarding inhibition of thrombogenicity. Furthermore, the degeneration of the grafts due to calcification should be minimized, which could lead to failure of the implant.

Beyond analysis of cellular repopulation and calcification of the implants, the persistence of laminin coating was investigated.

We generated the hypothesis that laminin as a graft coating agent of decellularized biological vascular prostheses potentially reduces immune response, calcification and intima hyperplasia.

2. Published manuscript

Toshmatova M., Nakanishi S., Sugimura Y., Schmidt V., Lichtenberg A., Assmann A., Akhyari P.

"Influence of Laminin Coating on the Autologous In Vivo Recellularization of Decellularized Vascular Protheses"
Materials 2019 Oct 15;*12*(20), 3351; doi;10.3390/ma12203351, PMID: 31618810

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Article

Influence of Laminin Coating on the Autologous In Vivo Recellularization of Decellularized Vascular Protheses

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Received: 18 August 2019; Accepted: 10 October 2019; Published: 15 October 2019

Abstract: Decellularization of non-autologous biological implants reduces the immune response against foreign tissue. Striving for in vivo repopulation of aortic prostheses with autologous cells, thereby improving the graft biocompatibility, we examined surface coating with laminin in a standardized rat implantation model. Detergent-decellularized aortic grafts from donor rats (n = 37) were coated with laminin and systemically implanted into Wistar rats. Uncoated implants served as controls. Implant re-colonization and remodeling were examined by scanning electron microscopy (n = 10), histology and immunohistology (n = 18). Laminin coating persisted over eight weeks. Two weeks after implantation, no relevant neoendothelium formation was observed, whereas it was covering the whole grafts after eight weeks, with a significant acceleration in the laminin group ($p = 0.0048$). Remarkably, the intima-to-media ratio, indicating adverse hyperplasia, was significantly diminished in the laminin group ($p = 0.0149$). No intergroup difference was detected in terms of medial recellularization ($p = 0.2577$). Alpha-smooth muscle actin-positive cells originating from the adventitial surface invaded the media in both groups to a similar extent. The amount of calcifying hydroxyapatite deposition in the intima and the media did not differ between the groups. Inflammatory cell markers (CD3 and CD68) proved negative in coated as well as uncoated decellularized implants. The coating of decellularized aortic implants with bioactive laminin caused an acceleration of the autologous recellularization and a reduction of the intima hyperplasia. Thereby, laminin coating seems to be a promising strategy to enhance the biocompatibility of tissue-engineered vascular implants.

Keywords: bioengineering; laminin; coating; decellularization

1. Introduction

Cardiovascular disease is the main cause of death globally [1]. In case of need for small-caliber arterial replacement, artificial implants have proven to be insufficient, and autologous grafts are frequently limited in terms of wall quality, availability, and long-term durability.

Particularly in the last decade, tissue-engineered large-caliber arterial and valvular grafts have presented good patency and mid-term durability in preclinical animal models as well as in humans [2–4]. For small-caliber arterial grafts, decellularization has been shown to improve their performance in small

animal models as well. Decellularization reduces the inflammatory response against non-autologous implants [5]. Depending on the agents that are used to obtain acellular scaffolds, the speed of cellular repopulation varies [6]. Implant coating with bioactive proteins can further accelerate the repopulation process [7–9]. Not only beneficial results, such as rapid re-endothelialization and cell migration into the media without any inflammatory reactions, were observed in coated grafts, but also adverse intima hyperplasia occurred [7,8].

Laminins are heterotrimeric glycoproteins of the extracellular matrix that especially occur in the basement membrane [10]. They can bind to other matrix molecules, thereby contributing to cell differentiation, cell shaping and migration, maintenance of tissue phenotypes and promotion of tissue survival [11]. Laminins are crucial components for basement membrane assembly, initiating the process by binding to surface receptors and receptor-like molecules. Furthermore, laminins participate in the assembly of the cytoskeleton, promoting cell migration, adhesion of epithelial cells and hemidesmosome formation by their cytoplasmic domains [12].

In our study, we aimed to accelerate the non-hyperplastic autologous in vivo recellularization of decellularized aortic grafts using laminin for biofunctional implant coating.

2. Materials and Methods

2.1. Animals

Male Wistar rats (200–250 g) from the animal care facility of the Heinrich Heine University (Duesseldorf, Germany) were used for all groups. All experiments were approved by the state animal care committee (reference number 84–02.04. 2012. A391) and conducted following the national animal welfare act.

2.2. Preparation of Donor Aorta and Graft Decellularization

Aortic graft harvesting (n = 37) was conducted as recently published [6]. In brief, donor rats were euthanized by isoflurane. Following thoracotomy, the aorta was dissected from surrounding tissue, before thorough antegrade and retrograde rinsing (phosphate buffered saline (PBS)) with 12.5 IU/mL heparin was carried out. Afterwards, a U-shaped aortic graft was prepared.

Harvested grafts were decellularized according to a recently published process using a protocol employing only biologically derived components and consisting of: 3 days of cycles with 50 nM latrunculin in glucose D-MEM, 0.6 M KCl, 1.0 M KI, 1 kU/mL DNase I in PBS and 3 cycles of 24 hours with 1% penicillin/streptomycin and 0.5% sodium azide. The protocol was performed in 15 mL tubes, filled with 6 mL per graft, containing up to 2 grafts. Supplemental Figure S1 displays a representative graft decellularized by this protocol.

2.3. Graft Coating with Fluorescent Laminin

Grafts (n = 23) were incubated in 1 mg/ml laminin (Sigma Aldrich, Taufkirchen, Germany) in PBS for 24 hours at 37 °C. After incubation, the grafts were shortly rinsed with PBS and transferred to implantation. In order to examine the persistency of laminin coating, implantations of grafts (n = 9) with laminin coupled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) were performed. For the coupling procedure, laminin solution (1 mg/mL in PBS) was added to a sodium bicarbonate buffer (1 M NaHCO3, pH 7.3) to a final concentration of 20 µM. Labeling was conducted in the presence of 20–fold molar excess of the fluorophore Alexa488 by incubation with rotation for 1 h at room temperature in the dark.

2.4. Graft Implantation

The implantation procedure was conducted as previously published [8]. Recipient rats were anesthetized by 2.0%–2.5% isoflurane inhalation, orally intubated, and central venous catheter insertion followed. After median laparotomy, the intestines were lateralized, and the abdominal aorta was

dissected from the inferior vena cava. Heparin was administered systemically (300 IU/kg), the aorta was clamped, and a U-shaped, decellularized aortic graft was anastomosed to the infrarenal aorta using an end-to-side technique and 10–0 monofilament, nonabsorbable polypropylene sutures (Ethicon, Norderstedt, Germany). Intermittent reperfusion guaranteed a minimization of the limb ischemia times. After release of graft blood flow, the abdominal aorta between the anastomoses was ligated for improved perfusion (Supplemental Figure S1B). After clinical observation during reperfusion for at least 10 min, particularly paying attention to the lower extremities perfusion, the abdomen was closed, and recipients recovered from anesthesia. Immediately after the implantation, Doppler sonography assessment of the implants was conducted to evaluate their function (Philips HDX11 ultrasonography system equipped with a 15 MHz probe, Philips, Amsterdam, Netherlands).

2.5. Graft Explantation

Grafts were explanted after 2 and 8 weeks from recipient rats anesthetized as described above. Laparotomy was performed, the abdominal aorta was cannulated, and the implanted grafts were rinsed with heparinized PBS, thoroughly excised, and further processed for scanning electron microscopy (SEM) (n = 10) or histology (n = 27). Aortic grafts were divided into four regions: proximal anastomosis (region A1), ascending aorta (region A2), descending aorta (region B1) and distal anastomosis (region B2) (Supplemental Figure S1). After 2 weeks in vivo, grafts were fixed in 2.5% glutaraldehyde solution for 1 h, washed in sodium chloride 0.9% solution 3 times for 5 min and dehydrated with ethanol (50%, 70%, 80%, 96%, 100%) for 5 min in each. After drying completely, the regions were gold-coated and then examined under a scanning electron microscope (Leo 1430 VP, Zeiss, Wetzlar, Deutschland).

2.6. Histology

Graft cryo-sections (5 µm) underwent histological assessment (hematoxylin/eosin staining, von Kossa staining, Movat's pentachrome staining). The amount of luminal neointima formation was examined by a standardized hematoxylin/eosin staining-based protocol as recently published. In brief, each graft was divided into four regions as described above, and three sections from each region were analyzed in eight segments divided by radial lines. In each segment, the percentage of neointima formation was determined. Similarly, in each of the eight graft wall segments, the media repopulation was evaluated as follows: The number of cells migrating to the implant media was counted. To measure the amount of adverse intima hyperplasia, the intima-to-media ratio was calculated in the same predefined areas and segments of the grafts as described above. In each segment, the thickness of the neointima and the media was measured to assess the intima-to-media ratio.

In order to determine the amount of calcification after explantation, cross-sections were stained using a standard protocol for von Kossa staining, and then subdivided into four pieces. The von Kossa score was calculated based on a scoring system representing the relative extent of von Kossa-positive areas, with intima values ranging from 0 to 3, while media calcification values ranged from 0 to 5 [13].

2.7. Immunohistology

Cryo-sections (5 µm) were incubated for 10 min with 0.25% triton-X and 1 h with 5% bovine serum albumin + 0.1% tween-20, in each case at room temperature. The primary antibodies were: anti-von Willebrand factor ((vWF), DAKO Hamburg, Germany), anti-alpha-smooth muscle actin ((aSMA), Sigma Aldrich, Taufkirchen, Germany), anti-CD3 (Sigma Aldrich, Taufkirchen, Germany), and anti-CD68 (Abcam, Cambridge, UK), each + 1% bovine serum albumin + 0.1% tween-20 for 1 h at 37 °C. Secondary antibodies conjugated to the fluorophores Alexa 546 and Alexa 488 (Invitrogen, Carlsbad, CA, USA) + 1% bovine serum albumin + 0.1% tween–20 were applied for 45 min under dark and humid conditions at 37 °C. Vectashield mounting medium containing DAPI (4',6-Diamidino-2-phenylindol) was used to cover the sections, and microscopy was conducted utilizing a DM2000 system with a digital camera DFC 425C (Leica, Wetzlar, Germany) and the Leica Application Suite V3.7 software.

2.8. In Situ Zymography

To evaluate the MMP (matrix metalloproteinase) activity in explanted grafts, in situ zymography was performed. Cryo-sections (5 µm) of explanted aortic grafts were incubated for 20 h at room temperature with fluorescein-labeled gelatin (40 g/ml; Invitrogen, Carlsbad, CA, USA) in 50 mM Tris-HCl, 10 mM $CaCl_2$, 150 mM NaCl and 5% triton-X. In order to examine the specificity of gelatinase activity, incubations with gelatin and 20 mM EDTA, only Buffer without gelatin and cis-ACCP solution (Cayman Chemicals, Ann Arbor, MI, USA) were conducted. Finally, sections were mounted with DAPI-containing Vectashield (Vector Labs, Peterborough, United Kingdom), and the MMP activity was visualized by fluorescence microscopy as described above. The MMP activity was assessed using Image J to measure the mean fluorescence intensity of the aortic graft wall.

2.9. Statistics

All continuous variables are presented as mean values ± standard errors of the mean. Student's t-tests were conducted for direct group comparisons. p-values lower than 0.05 were considered to indicate statistical significance. Data analysis was performed with GraphPad Prism v 6.01 (GraphPad Software, San Diego, CA, USA).

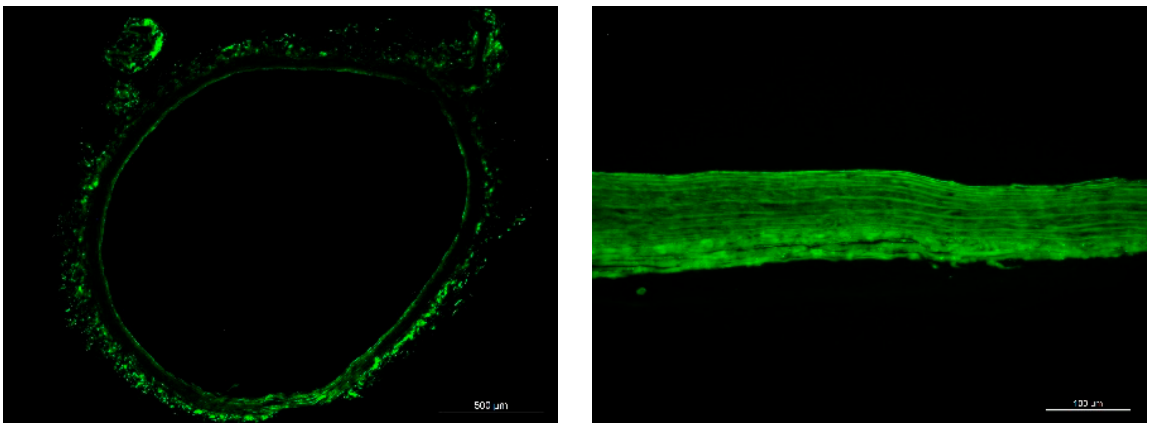
3. Results

3.1. Operative Outcome

The graft harvesting time amounted to 2.3 ± 3.5 min, while the graft implantation cut-suture time was 46.2 ± 5.5 min with infrarenal aortic clamping times ranging from 12 to 31 min (17.3 ± 4.6 min).

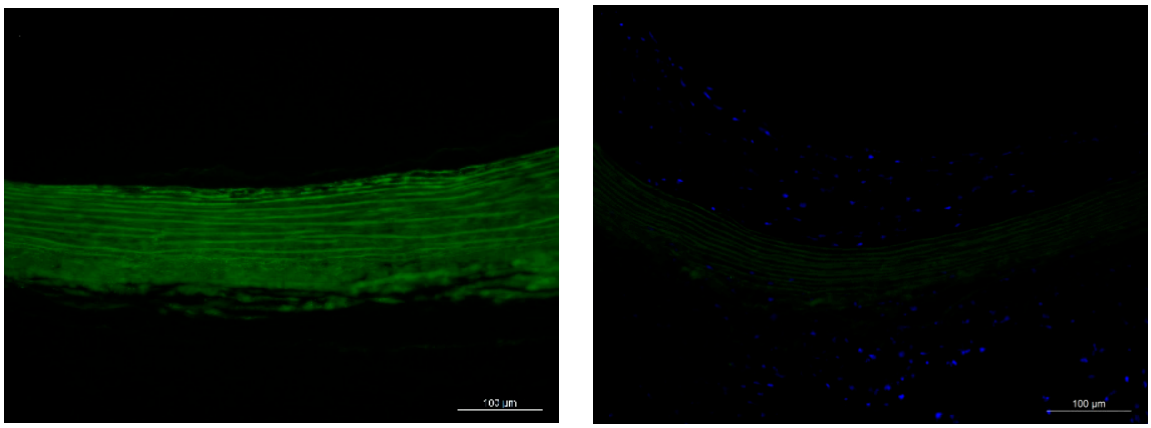
3.2. Laminin Coating Persistency

Alexa Fluor 488-coupled laminin coating resulted in a continuous, brightly green fluorescence along both surfaces of the graft. The fluorescence was found to be persistent after two and eight weeks, though the intensity was visually reduced after eight weeks (Figure 1).



(**A**) (**B**)

Figure 1. *Cont.*

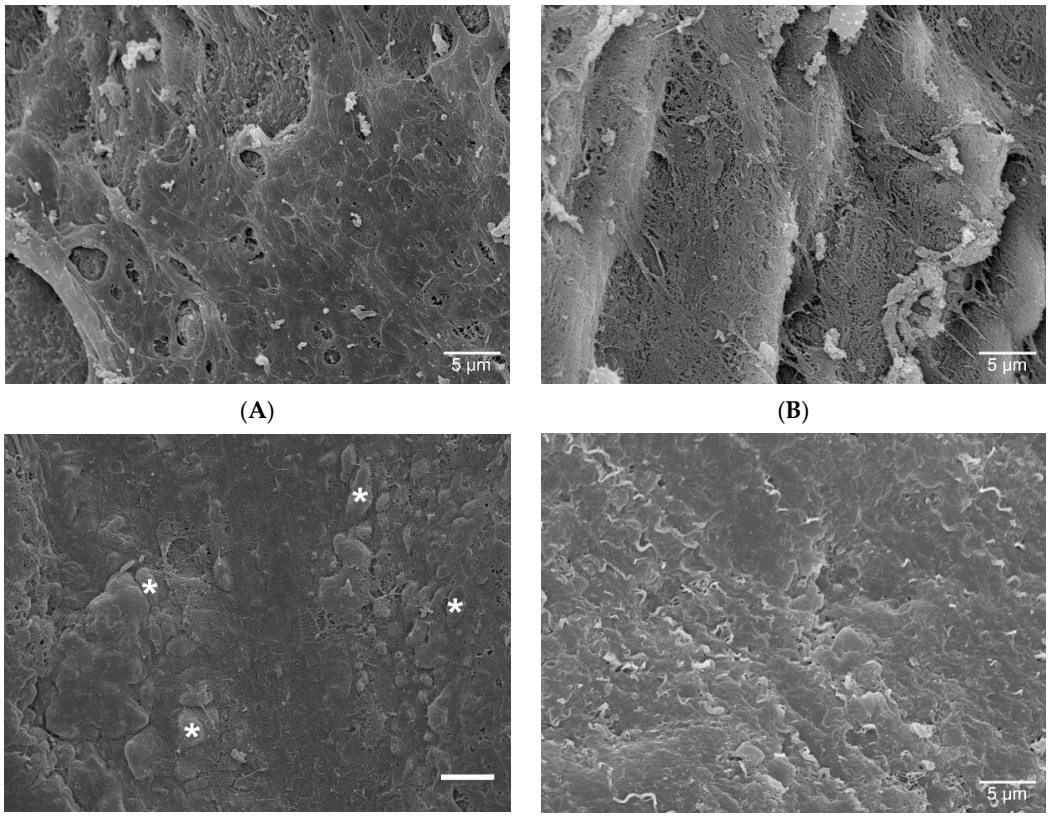


(**C**) (**D**)

Figure 1. Laminin coating resulted in bright green fluorescence (Laminin-bound Alexa 488) along both surfaces of the aortic grafts (**A**). Two weeks after implantation, green fluorescence was detected throughout the whole graft wall (**B**). By week 8, the intensity of the fluorescence had decreased (**C**) Control graft 8 weeks after implantation (**D**). Blue in D, DAPI (not stained in (**A**–**C**)). Scale bars = 500 µm in (**A**); 100 µm in (**B**–**D**).

3.3. Quantity and Quality of Neointima Formation

After two weeks, only extracellular matrix was detected on the luminal surface of SEM samples from explanted decellularized grafts, while autologous cellular colonization was not observed (Figure 2).



(**A**) (**B**)

(**C**) (**D**)

Figure 2. Representative SEM pictures of grafts after 2 weeks in vivo. Laminin group (**A**), Control group (**B**). Non-implanted controls: Native aorta (**C**) (exemplary asterisks on endothelial cells), and decellularized aorta (**D**). Scale bars = 5 µm.

Hematoxylin/eosin staining at 8 weeks showed neointima in the laminin group consisting of predominantly one layer or a completely restructured graft wall including fiber remodeling, mostly in A1 and B2 regions, whereas parts with multi-layer neointima were also observed. In the control group, neointima predominantly consisted of areas with multi-layer hyperplastic intima (Figure 3).

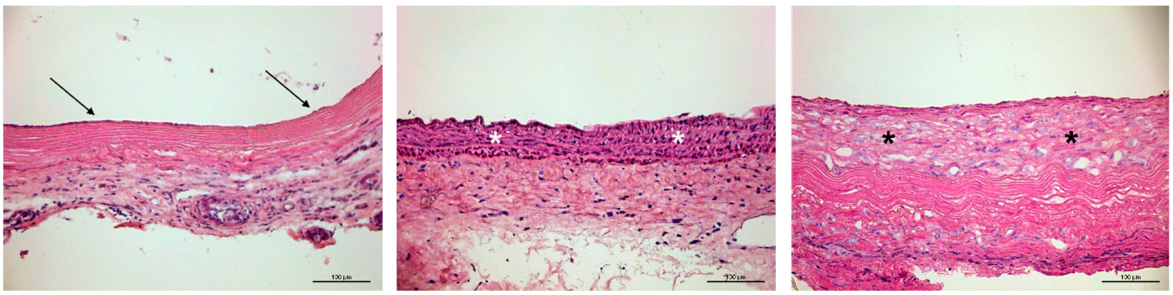


Figure 3. Cross-sections of grafts after 8 weeks in vivo. In the laminin group, the neointima predominantly consisted of one layer (arrows in (**A**)), and areas with restructured implant wall medially populated by autologous cells were observed (asterisks in (**B**)), whereas the neointima of the control group mostly exhibited intima hyperplasia (asterisks in (**C**)). Hematoxylin/eosin staining. Scale bars = 100 μm.

After 8 weeks in vivo, a continuous de novo cellular repopulation on the luminal side of decellularized conduits was observed, which was significantly higher in the laminin group (Percentage of re-endothelialized luminal surface: 98.4% ± 0.6% vs. 91.3% ± 3.1% in the control group, $p = 0.0048$).

For analysis of adverse hyperplastic neointima formation, the intima-to-media ratio was measured in explanted decellularized aortic grafts. Eight weeks after implantation, the overall intima-to-media ratio in the laminin group was significantly lower than in the uncoated controls (0.9 ± 0.1 vs. 1.5 ± 0.2, $p = 0.0149$) (Figure 4). In all subregions of the graft except in B1, the intima-to-media ratio in the laminin group was decreased as compared to controls.

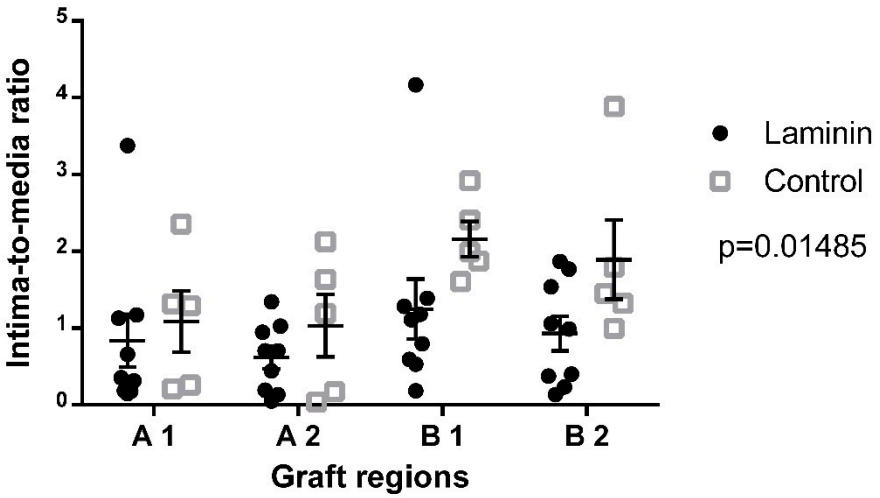


Figure 4. Semiquantitative analysis of the intima-to-media ratio 8 weeks after implantation showed significantly higher scores in the control group as compared to the laminin-coated group.

Immunofluorescence staining of decellularized aortic grafts 8 weeks after implantation revealed most of the cells in the hyperplastic intima areas of both groups to contain aSMA. The luminal neoendothelial layer stained positive for vWF (Figure 5A,B). By Movat's pentachrome staining, in hyperplastic intima areas, glycosaminoglycan-rich extracellular substance was detected around cells

with a fibroblastoid phenotype. In both groups, no inflammatory cell markers (CD3 for lymphocytes and CD68 for macrophages) were detected at 8 weeks (Figure 5C,D).

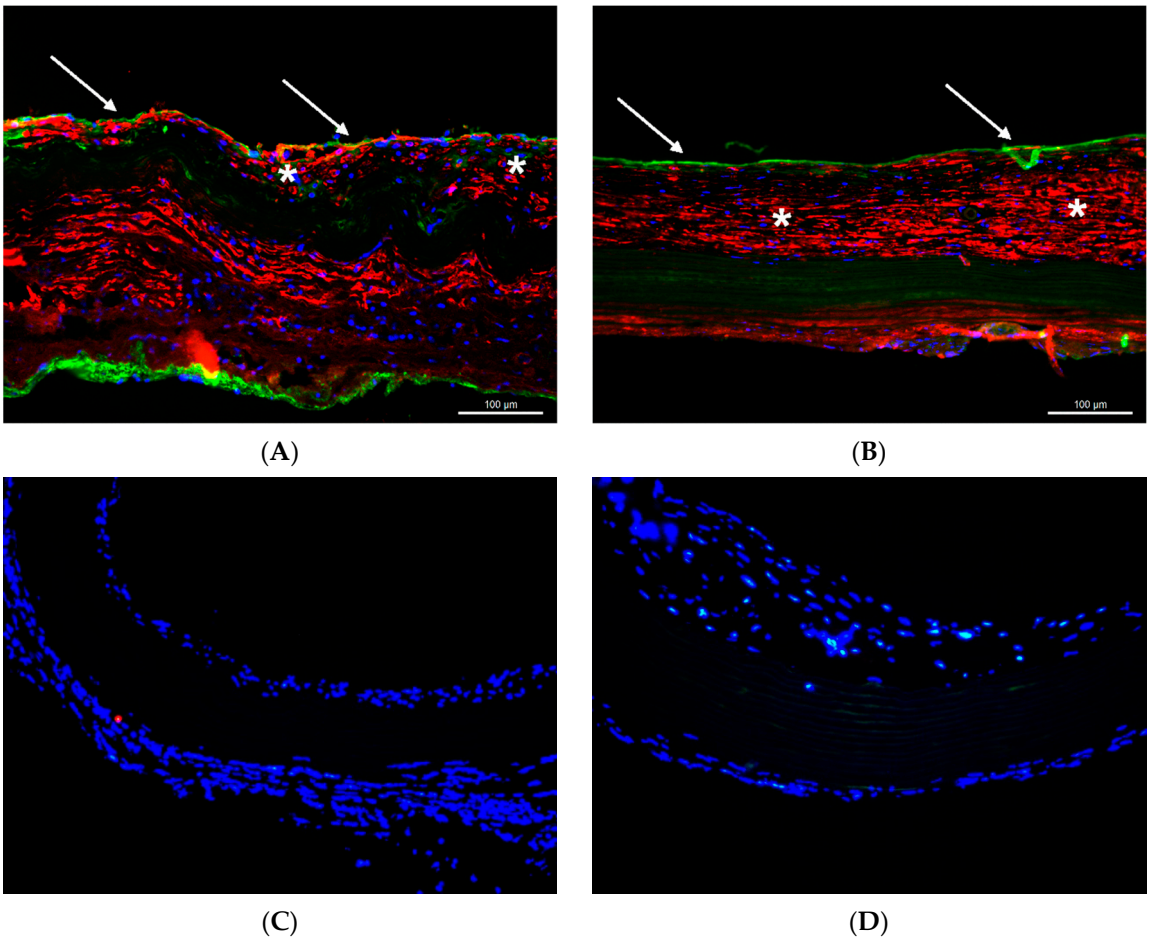


Figure 5. Representative sections through the ascending aorta of grafts after 8 weeks in vivo. In both groups (Laminin group: (**A**), Control group: (**B**)), the neointima (asterisks) was formed by aSMA-positive cells (red), and vWF-positive endothelial cells covered the luminal border (green, arrows). In both groups (Laminin group: (**C**), Control group: (**D**)), inflammatory markers did not stain positive for CD3 (red) or CD68 (green). Blue, DAPI. Scale bars = 100 µm in (**A**,**B**); 200 µm in (**C**,**D**).

After 8 weeks in vivo, von Kossa staining revealed small areas of microcalcification in the neointima and in the tunica media, and macrocalcification in the tunica media of the decellularized implants (Supplemental Figure S2). Remodeled regions of the tunica media with high content of aSMa-positive autologous cells did not exhibit hydroxyapatite deposition. The extent of calcification, as assessed by the previously established von Kossa score, was not significantly different between the laminin and control groups, neither in the neointima (0.3 ± 0.1 in the laminin group vs. 0.5 ± 0.01 in the control group, $p = 0.0661$), nor in the media (1.7 ± 0.2 vs. 1.1 ± 0.2, $p = 0.0779$) (Figure 6).

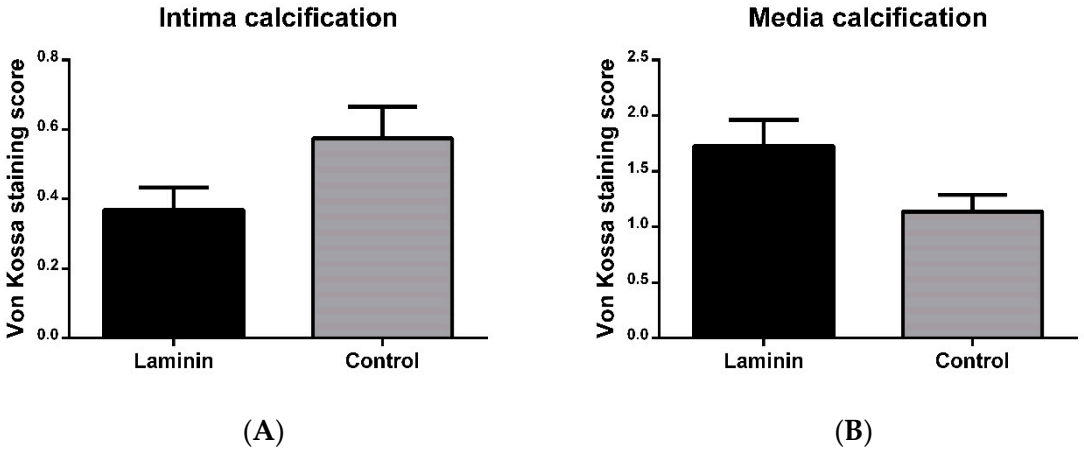


Figure 6. Semiquantitative analysis of intima (**A**) and media (**B**) calcification after 8 weeks in vivo. Neither in the intima nor in the media was the amount of calcification significantly different in laminin-coated versus uncoated control grafts.

3.4. Medial Graft Repopulation and Restructuring

The number of autologous cells repopulating the decellularized media was counted in all graft regions, and did not differ between the groups (Laminin: 127.6 ± 69.13 vs. Control: 242.2 ± 71.94 cells per cross-section, p = 0.2577). In the laminin group, remodeling of the media by autologous cells migration and extracellular matrix production was mostly observed in the A1 and B2 regions around the graft anastomoses, while in the control group, it was found more frequently only in the A1 region. The restructured media was full of fibroblast-shaped cells.

In situ zymography after 8 weeks showed MMP activity in the adventitia and predominantly in the neointima of decellularized aortic grafts. Areas of increased cell density, such as in hyperplastic neointima, exhibited a marked MMP activity, but there was no statistically significant difference between the two groups (p = 0.4170) (Figure 7).

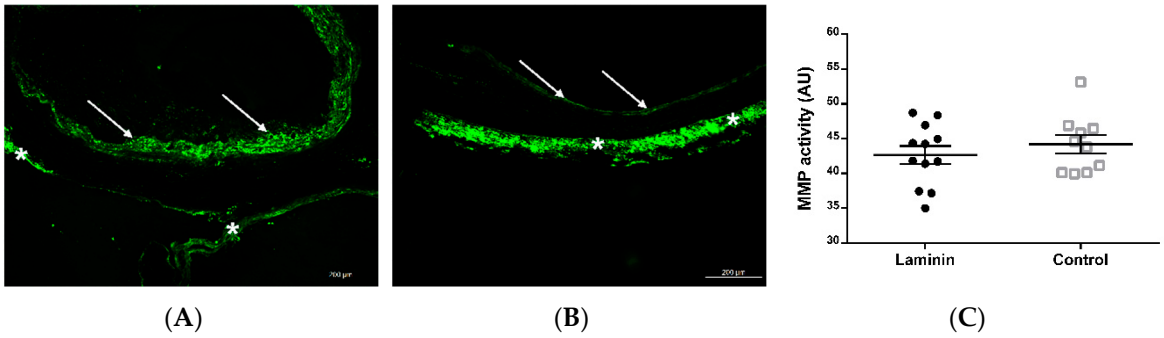


Figure 7. Representative cross-sections with in situ zymography after 8 weeks showed MMP activity (green) predominantly in the neointima (asterisks) and adventitia (arrows) in the control (**A**) and laminin (**B**) group, while semiquantitative analysis (**C**) did not show a statistically significant difference. AU = arbitrary units.

4. Discussion

In the present study, we report on the impact of laminin coating on the in vivo fate of decellularized aortic grafts. In particular, laminin coating accelerated the autologous cellular repopulation of the implants, while inhibiting adverse intima hyperplasia.

For interpretation of the study results, the durability of laminin coating is important. In earlier reports, there was no information on the persistency of laminin coating in vivo, whereas in our study, laminin-bound fluorescence was found to persist on decellularized grafts for at least eight weeks. The

green-fluorescent Alexa 488 coupled to the laminin proteins was initially observed along the surfaces of the grafts. However, after implantation in the blood circulation of rats, laminin spread through the whole graft wall, which may be influenced by blood pressure. Besides laminin movement through the tissue, separation and movement of the fluorophores may be considered as the only explanation for the observed distribution of the fluorescence signal over time. In this scenario, laminin might have been degraded earlier, while the fluorophore might persist in the graft wall. On the other side, laminin might persist to an even larger extent than was assumed from the detected fluorescence signal, since the fluorophore itself might have been degraded in vivo by enzymatic activity. Taken together, the kinetics of the in vivo degradation of fluorophores themselves as well as their uncoupling from bioactive proteins need to be further elucidated. Laminin antibody staining is not supportive in this context. We had previously conducted laminin antibody staining of decellularized and coated grafts, but all the grafts had stained positive, confirming data from other groups [6]. A reason for this issue may be the immunological relation of most laminin isoforms to laminin 111, since they contain either the β1 or the δ1 chains, or both. Therefore, antisera raised against laminin 111 purified from the EHS sarcoma stain all basement membranes, even in the absence of the laminin α1 chain. However, even in the case of potentially earlier degradation of laminin coating, its beneficial effects on graft repopulation have been observed during the follow-up until week 8.

In our study, laminin significantly accelerated the de novo endothelialization of decellularized grafts. The potential of laminin to induce the adhesion of circulating progenitor cells has been discussed previously. In vitro studies have shown that the human laminin α2 large globular 1 domain exhibits cell adhesion activity and binds to syndecan-1, which was proven in the cultured PC12 cell line from transplantable rat pheochromocytoma [14]. Laminin and laminin-derived peptides promote cell adhesion also in dental implants in vitro [15], and laminin-derived peptides contribute to the in vivo regeneration of peripheral nerves in rats. The re-endothelialization of acellular implants coated with laminin begins within the first two weeks after implantation, whereas relevant amounts of cellular population occur between week 2 and week 8. These data are in line with findings from previous cardiovascular graft implantation studies in the herein applied rat model [7].

In the present examination, laminin did not only accelerate re-endothelialization, but also decreased adverse neointima hyperplasia in decellularized aortic grafts. The anastomoses regions underwent an early remodeling process with high autologous cell migration activity, supporting the hypothesis that implant cellularization starts predominantly from the anastomoses parts as previously described [7,15,16]. The neointima areas were populated by aSMA-positive myofibroblasts, and none of the restructured parts of the grafts stained positive for inflammatory markers, implicating the invading cells most likely to be activated fibroblasts originating from the native aorta, and excluding relevant inflammatory response against the implants.

A previous in vitro study has reported on the migration-promoting activity of laminin isoforms in tumor cells [17]. Nevertheless, laminin surface coating of decellularized grafts did not significantly increase medial repopulation in the whole grafts after eight weeks in vivo, though aSMA-positive cells invasion was observed in the areas of media fiber restructuring. Since the repopulation of the media occurred mostly in the anastomotic parts and barely in remote parts of the grafts, longer follow-up periods may be necessary to observe complete media repopulation.

In spite of beneficial effects of laminin on neointima formation, including a trend towards reduced intima calcification, we could observe a tendency of laminin-coated grafts to develop media calcification. In this context, in vitro studies have indicated that laminin-1 can selectively recruit osteoprogenitors through an integrin β1-dependent cell attachment effect [18]. Moreover, previous in vivo examinations reported that laminin stimulates osteointegration [19]. The areas of wall restructuring did not show any calcification, whereas parts far from the anastomoses were more susceptible to calcification, which may be due to enhanced calcium binding to the extracellular matrix, particularly to damaged elastin fibers [20], in the absence of matrix-producing cells. Therefore, further strategies are necessary to promote an acceleration of autologous media repopulation, which may be addressed by cell-attracting

agents as well as modified decellularization protocols resulting in a graft matrix architecture that favors cell migration [6].

5. Conclusions

Laminin coating significantly accelerated the non-inflammatory autologous in vivo recellularization and decreased the adverse neointima hyperplasia of decellularized aortic grafts. In this regard, laminin coating seems to be a promising strategy to improve the biocompatibility of tissue-engineered vascular implants, whereas a combination with alternative bioactive coating agents might further improve the outcome, particularly in terms of long-term calcification of the grafts.

Supplementary Materials: The following are available online at http://www.mdpi.com/1996-1944/12/20/3351/s1, Supplementary Figures S1 and S2.

Author Contributions: Conceptualization, A.A., P.A., M.T.; methodology, A.A.; software, M.T.; validation, A.A., P.A. and A.L.; formal analysis, M.T.; investigation, S.N., Y.S., V.S.; resources, A.L., P.A.; data curation, M.T.; writing—original draft preparation, M.T.; writing—review and editing, A.A. and P.A.; visualization, M.T.; supervision, A.A., P.A.; project administration, A.A.; funding acquisition, A.A.

Funding: The project was partially funded by DAAD (German Academic Exchange service).

Acknowledgments: The authors gratefully acknowledge Steffen Köhler (Centre for Advanced Imaging; Heinrich Heine University Duesseldorf) for scanning electron microscopy.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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3. Discussion

Tissue-engineered grafts can be the future at re-revascularization operations. Decellularized grafts are good candidates to improve clinical results for cardiovascular implants, already presenting with promising outcome in case of valvular grafts [19]. The decellularization protocol has shown gut results of removing all cells and ECM integrity. Using enzymes to remove DNA is important to complete the decellularization [20]. Accelerated recellularization and decreased intima hyperplasia *in vivo* alongside with lower degeneration by calcification and thrombogenicity are proved achievements of tissue engineering [15].

In our experimental work, we used decellularized allografts from rats and laminin as luminal coating agent. Using fluorescence microscopy, we have seen laminin coating on the internal and external surface. After 2 and 8 weeks, we could observe fluorescence throughout the whole graft wall, which can mean that blood flow has distributed the laminin in the whole wall of the grafts. The intensity of laminin signal decreases over the time, probably by laminin being replaced due to cell migration and being washed away with the blood stream. After two weeks, no recellularization was observed with electron microscopy, however more investigation such as histology and molecular examination could be useful to conclude on the actual status of the implanted grafts. This could help us to evaluate the time course of recellularization and give us a hint on degeneration. We could show that the laminin coating significantly accelerates the *in vivo* recellularization of the grafts in 8 weeks observation. Moreover, the recellularization begins presumably from the parts around the anastomoses, and the migration through the grafts continues over time. Probably, more time than 8 weeks is needed to repopulate the whole media.

The role of laminin in tissue formation, as well as cell integration, has been shown in a mouse model: the absence of encoding gene of laminin has resulted in early embryonic lethality due to failure of the basement membrane [21]. The implanted graft has undergone remodeling, what implied the function of laminin in the development of tissues. Laminin is an important part of the ECM that creates a scaffold for cell attachment and contributes to the reorganization of

wounded tissues and implants [22], [23]. This underlines the potential of the ECM protein as coating agent for graft biofunctionalization [14], [15]. The beneficial influence of laminin on intima hyperplasia and intima remodeling has not been reported before. But we have seen that laminin due to being persistent on the luminal surface, significantly provides cell migration and remodeling of the graft.

Zaharia et al. showed that laminin as coating agent significantly affected myoblast migration on nanofiber scaffolds. Moreover, in combination with genipin, proliferation of myoblasts could be also observed [24]. The cell-adhesive effect of laminin was also proved in previous work. Shortly, PDMS was coated with laminin, and then *in vitro* and *in vivo* experiments were conducted to compare PDMS and PDMS with O_2 plasma treatment. The coating with laminin resulted not only in better attachment of epithelial cells, but also in better hydrophilic property of PDMS [25].

The graft degeneration has been also avoided in our experiment of laminin coating of vascular grafts. There is no calcification in any remodeled part of the grafts. Bougas et al. examined laminin-1 as coating agent for polished cylindrical hydroxyapatite implants in the rabbit tibiae and have shown an increased amount of supporting bone around the implant resulting in good micromechanical properties [23], [26]. On the contrary, our study has not revealed higher calcification risk in the laminin group, and the neointima was covered with vWF-positive endothelial cells.

Unfortunately, there is not much evidence on laminin being used as coating agent in cardiovascular surgery yet, particularly not in comparison to other bioactive peptides. In this context, the current study provides new insights into vascular graft biofunctionalization by laminin coating.

3.1. Perspectives

Decellularization provides ECM grafts with improved biocompatibility that may substitute common clinical standard implants. Coating agents, which have been shown as useful in tissue engineering approaches *in vitro* and *in vivo*, allow

targeted graft modification and improvement of their biocompatibility. Laminin coating has been shown to be effective in small diameter vessel grafts and can be combined with other coatings to optimize the outcome. Particularly, laminin should be examined as luminal coating agent to accelerate the recellularization of all parts of the graft in long-time experiments. These data need to be further evaluated before clinical application may be considered.

All experiments were conducted according to the national animal welfare act and approved by the state animal care committee (reference number 84-02.04. 2012.A391).

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5. Note of thanks

I sincerely thank my supervisor PD Dr. med. Alexander Assmann for the inspiration, belief and being fair referee during the whole research time. Without him, I could not start anything in the experimental surgery and be successful.

I thank the chief of the Clinic of Cardiac Surgery, Prof. Dr. med. Artur Lichtenberg, for the opportunity he gave me to improve myself, for the trust and for showing all the ways to being successful in team. My pleasure and greetings to Prof. Dr. med. Payam Akhyari for the support and being there at all steps of research time with profiting advices and comments. Without them the work could not be finished.

I thank also all my co-authors and coworkers from the experimental surgery of the clinic for all their support, work and patience they have had for me.

Special thanks to my husband Akmal for being there all the time and the enormous support he sacrificed for me. Thanks for my lovely daughter Amina for her laugh, which kept me always going on.