Complications and grafts in septic vascular surgery

Analysis of the thrombogenicity properties of cryopreserved allografts and the determination of the healthy vascular microbiota

Ph.D. thesis

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1 Introduction

Managing vascular infections presents one of the significant challenges in vascular surgery. The key to treating an infected surgical site, in the absence of a suitable autologous vessel, lies in utilizing an infection-resistant, biocompatible, durable, and readily available material. However, the optimal graft material remains unavailable, although cryopreserved allografts show promise. Despite their advantages and increased production levels, allografts are still associated with numerous complications, including bleeding, graft degeneration, occlusion, or aneurysm formation. Several factors influence the success of vascular allograft transplantation, among which production methods play a crucial role. Cryopreservation, involving freezing and thawing, poses potential risks to grafts, and its advantages and disadvantages require further elucidation. Additionally, our understanding of donor-recipient interactions remains incomplete, with available data often contradictory. Studying the healthy vascular microbiota could provide insights into donor-recipient responses, reduce infection transmission risks during transplantation, and enhance our understanding of vascular diseases with unknown aetiologies.

2 Objectives

In this work, we analyse the direct and storage time-dependent effects of cryopreservation on the thrombogenicity of arterial allografts. In addition, we analyse the human vascular microbiota by examining arterial allografts as healthy vascular tissues.

I. Do cryopreservation and storage time alter the thrombogenicity of arterial allografts?

- fibrin deposition to the arterial wall
- platelet adhesion to the arterial wall

Hypothesis I.: The cryopreservation does not affect the thrombogenicity of arterial allografts.
Hypothesis II.: The six months storage time of cryopreservation does not affect the thrombogenicity of arterial allografts.

II. Does healthy human vascular tissue have a unique microbiota?

- developing a method for analysing the human vascular tissue microbiota
- characterising the healthy composition of the human vascular tissue microbiota

Hypothesis III.: Healthy human vascular wall contains bacterial hereditary material.
Hypothesis IV.: Healthy human arterial wall has a unique microbiota.

3 Methods

In our prospective studies, eleven human femoral arteries were analysed for thrombogenicity, and fourteen human femoral arteries were examined to determine the healthy human vascular microbiota. The arteries were harvested from eleven and fourteen, respectively, different donors in multi-organ donations between October 2019 and February 2021 in Hungary. Samples for thrombogenicity testing were examined at five time points: before the CP as a native sample (BC) and after the CP immediately (C0), on the first (C1), twelfth (C12) and twenty-fourth (C24) week of storage after CP, while native samples were used for the microbiota assay.

3.1 Measurement of thrombogenicity by immunofluorescence imaging

The flow-chamber model was used for testing the allograft samples as thrombogenic surfaces. Frozen cross-sections (10 μm) of allograft samples were perfused at 0.5 ml/min flow rate

with heparin-anticoagulated blood collected from healthy volunteers. The shear rate at the surface of the section was chosen as an adequate model of the rheological situation in medium-sized arteries, where deposition of both platelets and fibrin is enabled. Before the perfusion, the sections were blocked with 2 w/v% bovine serum albumin (BSA) in 0.05 M Tris buffer pH 7.4 containing 0.1 M NaCl and 0.02 w/v% NaN3 (TBS) for 45 minutes, and the 90-s perfusion was followed by a 30-s wash with 1.5 mM KH2PO4, 8.1 mM Na2HPO4 buffer pH 7.4 containing 137 mM NaCl and 2.7 mM KCl (PBS).

After that, the sections were fixed in acetone at 4˚C for 10 minutes, and the deposited platelets and fibrin were double-stained for indirect immunofluorescence microscopy. Sections were blocked with BSA-TBS for 30 min, followed by platelet staining using the mouse monoclonal antibody against human GpIIb/IIIa (4 μg/ml in BSA-TBS for 30 min, sc-53417, Santa Cruz Biotech), followed by three times 5 min washes in TBS, and 30 min incubation with the Goat-anti-Mouse IgG-Alexa Fluor 633 secondary antibody (2 μg/ml in BSA-TBS, Invitrogen, Budapest, Hungary). Double-staining was continued with the fibrin staining after three times 5 min washes in BSA-TBS: 30 min incubation with the primary rabbit polyclonal antibody developed against the N-terminal part of the gamma chain fibrin(ogen) (20 μg/ml in BSA-TBS, PA5-29734, Invitrogen), followed by three times 5 min washes in TBS, and 30 min incubation with the Goat-anti-Rabbit IgG-Alexa Fluor 546 secondary antibody (2 μg/ml in BSA-TBS, Invitrogen). Following the final washes in TBS, the stained sections were covered in 50% glycerol in TBS. Confocal images were taken from the slides using a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) using 488 nm, 543 nm and 633 nm excitation laser lines, respectively. Emissions were detected in the ranges of 500–530 nm, 565–585 nm and 650–690 nm, respectively. Each allograft vessel at each sampling

time point was perfused in duplicates or triplicates, and depending on the section size, 5–10 different images were taken of each perfused cryosection to survey the whole cross-sectional area of the vessel. Quantification of platelet and fibrin(ogen) coverage of the vessel wall was performed with the Image J software (NIH, Bethesda, MD, USA), selecting the region of interest, calculating its surface area in pixels and setting a threshold intensity value for automatic identification of platelets or fibrin(ogen) covered areas in percentage.

3.2 Identification of healthy human vascular microbiota: Deoxyribonucleic acid isolation, 16S ribosomal ribonucleic acid gene library preparation and MiSeq sequencing

After enzymatic dissolution, deoxyribonucleic acid (DNA) isolation was performed using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). The isolated DNA samples were then stored at -80°C until further processing for PCR amplification. The concentration of genomic DNA was measured using the Qubit2.0 Fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For amplification of bacterial DNA, PCR was conducted using tagged primers that targeted the V3-V4 region of the bacterial 16S rRNA gene. PCR and DNA purification steps followed Illumina's protocol. The PCR product libraries were evaluated using the Agilent 2100 Bioanalyzer with the DNA 1000 Kit (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of libraries were pooled and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3 (600 cycles PE). Extraction-negative controls and PCR-negative controls were included in each sequencing run to assess the potential contribution of extraneous DNA from reagents. Each vascular tissue sample was independently subjected to DNA extraction to ensure reproducibility. All analysis procedures

were performed in triplicate to minimise false results such as contamination using three different samples from each donor. The raw sequencing data were obtained from the Illumina BaseSpace and analysed using the CosmosID bioinformatics platform (CosmosID Metagenomics Cloud, CosmosID Inc. Rockville, MD, USA). The resulting data included taxonomic names, operational taxonomic unit IDs, frequency, and relative abundance.
The results of the current vascular samples were compared with the DNA amounts obtained from faecal and nasopharyngeal samples in our previous studies. The DNA isolation and 16S rRNA method were consistent across these studies.

4 Results

4.1 Thrombogenicity

4.1.1 Fibrin deposition to the arterial wall

The generation of fibrin was evaluated quantitatively by measuring the percentage of area covered by fibrin in the intima, media, and adventitia layers of immunofluorescent images of the graft cross-sections. The statistical analysis showed no significant increase in fibrin deposition in the cryopreserved samples compared to the fibrin levels detected in the native (BC) samples across any of the three layers. Notably, all significant differences between cryopreserved and native graft samples indicated a decrease in fibrin generation (out of the twelve sets of CP data, three median, three bottom quartile, and six top quartile values were lower than their BC counterparts). CP also tended to homogenise the potential for fibrin generation across the artery wall layers, with narrower IQRs observed in eight out of the twelve CP data sets.
To investigate the hypothesis that storage time affects fibrin deposition in cryopreserved grafts, we conducted a regression analysis of changes in fibrin coverage across the three arterial

wall layers over time. In seven out of the nine hypothesis tests performed, which examined the temporal trend of fibrin coverage in the three quartiles of the wall layers, a non-negative trend was excluded at a significance level of 0.05. In the remaining two cases, namely the lower coverage quartile of the media and intima layers, there was a probability of a non-negative trend of 20-22% and 14-28%, respectively. Therefore, a robust statistical analysis of fibrin coverage data throughout the twenty-four-week storage of arterial grafts definitely rules out the possibility of an increase in graft thrombogenicity over time and strongly supports a decrease in fibrin generation.

4.1.2 Platelet adhesion to the arterial wall

Platelet adhesion was evaluated by measuring the percentage of area covered by the GpIIb/IIIa-related immunofluorescence signal in the intima, media, and adventitia layers. Like fibrin deposition, the platelet coverage data within the IQR of the cryopreserved grafts largely overlapped with the corresponding range of the BC samples. However, unlike fibrin, in three out of the twelve datasets of cryopreserved samples, either all three (C0 and C1) or two (C12) quartile values of the media significantly exceeded the platelet coverage level of the native samples (BC). In the early cryopreserved samples of the intima (C0, C1, C12), there was a significant upward shift in the median of platelet coverage. However, a significant change in the upper and lower quartiles did not accompany this difference. Based on the data measured at week twenty-four, the values for all three layers no longer exceeded the values of the native sample, and significantly lower quartile values were obtained for the adventitia.

Regression analysis of the changes in platelet coverage across the three arterial wall layers over time excluded a non-negative trend at a significance level of 0.05 in seven of the nine hypothesis tests performed on the platelet coverage of the three wall layers' three quartiles. The two exceptions were the median

and the lower coverage quartile of the intima, where the probability of a non-negative trend was 5-11% and 7-18%, respectively. Therefore, the regression analysis of the platelet coverage data throughout the twenty-four-week storage of arterial grafts definitely rules out the possibility of a time-dependent increase in graft thrombogenicity and strongly supports a decline in platelet adhesiveness.

4.2 Healthy human vascular microbiota

In total, forty-two samples from fourteen patients were tested. The median of isolated DNA from vascular samples was 27.8 ng/μL (IQR: 21.4 ng/μL). From this starting amount, which also contains human DNA, after 16S rRNA PCR, a median DNA amount of 1.546 ng/μL (IQR: 0.762 ng/μL), after indexing PCR, a median DNA amount of 3.947 ng/μL (IQR: 1.996) was amplified. The average length of index PCR products was 667 base pair (bp) (standard deviation (SD): 55 bp). Negative controls and transport buffers did not yield measurable amounts of DNA after DNA isolation or 16S rRNA PCR. 5.8 million valid sequences were obtained, resulting in 3.9 million high-quality reads. One sample's median number of reads was 79,485 (IQR: 24,511).

The most prevalent phyla in the human vascular microbiota were *Proteobacteria* (31.78%), *Firmicutes* (29.18%), and *Actinobacteria* (23.05%).

At the genus level, the most abundant taxa in the human vascular microbiota were *Staphylococcus*, *Pseudomonas*, *Corynebacterium*, *Bacillus*, *Acinetobacter*, and *Propionibacterium*.

The median Chao1 alpha diversity of genera was 217.5(IQR: 107), indicating the diversity of bacterial genera. Among the bacterial taxa indirectly associated with atherosclerosis development, *Porphyromonas gingivalis* was found in two samples (0.54%, 0.16%), *Prevotella nigrescens* in one sample (0.1%), and all samples contained varying amounts of

Enterobacteriaceae spp with a median of 2.25% (IQR: 1.46). Comparatively, bacteria more commonly found in the intestinal flora of healthy individuals than in patients with atherosclerosis included *Roseburia*, which was found in eight samples (median: 0.13%, IQR: 0.08), and *Ruminococcus*, which occurred in thirteen samples (median: 3.07%, IQR: 1.78). *Helicobacter pylori* and *Chlamydophila pneumoniae*, bacteria directly implicated in the development of atherosclerosis, were not detected in any of the samples.

To confirm its uniqueness, the human vascular microbiota was compared with human stool and nasopharyngeal samples. Based on the Jaccard Beta Diversity PCoA analysis (p=0.001), we found significant differences between the samples, suggesting notable distinctions in the microbial composition between the vascular system and the gastrointestinal tracts.

The composition of the vascular microbiota did not show significant differences when compared by age, sex or smoking habits, as observed in the Three-dimension PCoA pictures. However, significant differences in the Chao1 alpha diversity of microbiota were found between the O and A blood groups (p=0.0106) and between the O and B blood groups (p=0.045) using the Wilcoxon Rank Sum test. The beta diversity of the microbiota was assessed using the Jaccard distance measure, and the PCoA biplot was utilised for visualisation. PERMANOVA analysis revealed significant differences in the Jaccard beta diversity values of the microbiotas between the O and A blood groups (p=0.003), between the O and B blood groups (p=0.037), and between the A and B blood groups (p=0.045).

5 Conclusions

5.1 Thrombogenicity

Our results showed that when arterial allografts were cryopreserved and stored for up to 6 months, their haemostatic

potential remained within the variability range of the fresh native arterial wall. In addition, the fibrin production and platelet adhesion of cryopreserved arterial allografts also decreased over time, minimising the risk of thrombotic occlusion of the grafts. Thus, the CP method showed a dual clinical benefit: retained haemostatic and reduced thrombogenic potential of the grafts. The only transient prothrombotic change was observed in the media of cryopreserved arterial allografts, where platelet deposition exceeded that of fresh native grafts during the first twelve weeks after CP. Antiplatelet treatment may be justified to prevent thrombotic occlusion if grafts are used during this early storage period. The markedly favourable trend of changes in the thrombogenicity of grafts over the six-month follow-up period of the current study justifies a longer-term study of the storage period of cryopreserved arterial allografts to optimise their thrombogenicity.

Thesis I.: The cryopreservation affects the thrombogenicity of arterial allografts. Although there was no difference in fibrin deposition between native and cryopreserved-immediately-thawed samples, however a prothrombotic change was observed in the media of cryopreserved arterial allografts, where platelet deposition exceeded that of fresh native grafts.

Thesis II.: The six months storage time of cryopreservation affects the thrombogenicity of arterial allografts. The fibrin production and platelet adhesion of cryopreserved arterial allografts decreased over time.

5.2 Healthy human vascular microbiota

Our present investigation is the first methodological description suitable for studying the healthy vascular wall microbiota. Based on it, the human arterial wall has a unique microbiota, which differs significantly in composition from other microbiota of the human body. The most important genera are *Staphylococcus*,

Pseudomonas, *Corynebacterium*, *Bacillus*, *Acinetobacter* and *Propionibacterium*, which occur in variable but dominant abundance in all vessel wall samples. In addition, donors were divided into distinct vascular microbiota clusters according to the ABO blood grouping system, suggesting vascular microbiota diversity between individuals with different antigenicity. The pathological or protective role of the microbiome in the background of various diseases can only be demonstrated if we know the microbiota associated with the healthy vascular wall. Our present study provides a basis for future research investigating the direct role of the microbiota in vascular wall abnormalities and the success of allograft transplantation. (92)

Thesis III.: Healthy human vascular wall contains bacterial hereditary material. Our methodology using 16S rRNA sequencing of the arterial samples from brain-dead donors successfully identified bacterial hereditary material in healthy human vascular walls in strict compliance with sterility rules.

Thesis IV.: Healthy human arterial wall has a unique microbiota. Compared to physiological faecal and nasopharyngeal microbiota examined using the same gene sequencing methodology as ours, the microbiota of the healthy vascular wall is significantly different.

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