Cardiology in the Young

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Staphylococcus aureus adheres avidly to decellularised cardiac homograft tissue in vitro in the fibrinogen-dependent manner

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Original Article

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Abstract

Objective: Infective endocarditis remains a severe complication associated with a high morbidity and mortality in patients after heart valve replacement. Exploration of the pathogenesis is of high demand and we, therefore, present a competent model that allows studying bacterial adherence and the role of plasma fibrinogen in this process using a new in-house designed low-volume flow chamber. Three cardiac graft tissues used for pulmonary valve replacement have been tested under shear conditions to investigate the impact of surface composition on the adhesion events. *Methods:* Tissue pieces of cryopreserved homograft (non-decellularised), decellularised homograft and bovine pericardium patch were investigated for fibrinogen binding. Adherence of *Staphylococcus aureus* to these graft tissues was studied quantitatively under flow conditions in our newly fabricated chamber based on a parallel plates' modality. The method of counting colony-forming units was reliable and reproducible to assess the propensity of different graft materials for bacterial attachment under shear. *Results:* Bacterial perfusions over all plasma-precoated tissues identified cryopreserved homograft with the lowest affinity for *S. aureus* compared to decellularised homograft presenting a significantly higher bacterial adhesion ($p < 0.05$), which was linked to a more avid fibrinogen binding ($p < 0.01$). Bovine pericardial patch, as a reference tissue in this study, was confirmed to be the most susceptible tissue graft for the bacterial adhesion, which was in line with our previous work. *Conclusion:* The two studied homograft tissues showed different levels of bacterial attachment, which might be postulated by the involvement of fibrinogen in the adhesion mechanism(s) shown previously for bovine tissues.

Infective endocarditis remains a diagnostic and therapeutic challenge associated with a high morbidity and mortality in patients after right ventricular outflow tract valve replacement.[1] A recent study reported a 5-year cumulative mortality of >50% in healthcare-associated endocarditis,[2] which is much higher than the mortality associated with many cancers. Clinical observations, which compared the common right ventricular outflow tract prostheses suggest a higher risk of infective endocarditis in patients after implantation of bovine jugular vein conduits than receiving a cryopreserved homografts, occurring at a rate of 3% per patient year.[3,4] Furthermore, the use of decellularised pulmonary homografts for pulmonary valve replacement in comparison to "non-decellularised" ones have shown very promising haemodynamical results, with nearly 100% of patients free of endocarditis at 10 -year follow-up and a significantly better freedom from re-operation.[5,6] The underlying mechanisms leading to a different susceptibility for infective endocarditis in different cardiac graft tissues are not known yet.

Staphylococcus aureus is amongst the most common cause of endocarditis.1 The onset of the disease requires bacterial adhesion to the valve endothelium leading to a proinflammatory endothelial cell phenotype, platelet recruitment and activation of the coagulation system.

Aside from the bacterium *per se*, the pathogenesis of endocarditis involves multiple host factors including tissue damage, inflammation and deposition of platelets and host matrix proteins such as fibrinogen/fibrin, to mention a few. Together these biological responses promote local colonisation by circulating bacteria during boosts of transient bacteraemia. Histological observations raise a concern that fibrin depositions occur on prosthetic valve leaflet tissue even without an ongoing infection.[7,8] As the native valve endothelium is resistant to bacterial adhesion, endothelial lesions along with the demonstrated fibrin depositions might be a critical precursor of bacteria and platelet attachment. It has been shown that the plasma protein fibrinogen and the platelet receptor $\alpha_{IIb}\beta_3$ mediate *S. aureus* adhesion to bovine jugular vein tissue.[9] As the surfaces of the grafts display different binding affinities for proteins, like fibrinogen,[10] the decellularisation of the homograft might lead to altered adsorption of plasma proteins. Fabricating a

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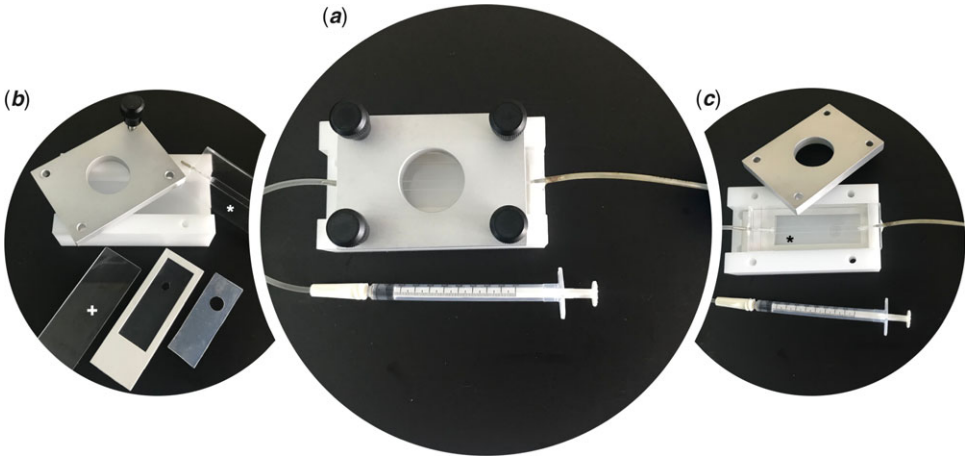

Figure 1. Schematic presentation of a newly developed low-volume parallel plate flow chamber (in-house design by the Centre for Molecular and Vascular Biology and fabrication by the Department of Cellular and Molecular Medicine, KU Leuven, Belgium). (***a***) A mounted flow set of dimensions LxWxH: 90 × 50 × 25 mm. (***b***) Elements of the flow chamber, which are placed in a bottom white frame as presented in (***c***) a top view. In brief, a thin foil slide with a white frame has a 4 mm circular perforation to allow the exposure of the tissue to the bacterial suspension. The slide has a recess for fitting a rubber gasket with an 8-mm whole, which serves as a holder in which the tissue piece is immobilised during the perfusion. A plastic microscopic slide (+) is used as an additional support for the setup and is placed underneath the tissue holder in the bottom part of the white chamber. The asterisk (*) presents an insert made of a Plexiglas that carries a channel flanked by metal inlet and outlet adaptors connected to the rubber tubes. It is placed on the top of the tissue holder to form a leak-free flow channel right above the central part of the tissue graft.

decellularised pulmonary homograft aims to increase its biocompatibility and to cause less activation of the immune system by removing almost all donor DNA during the decellularisation process.[11] In the months following implantation, prosthetic grafts become progressively re-endothelialised by new host cells,[12] of which the course is crucial in terms of the biocompatibility of the valves. The risk of infective endocarditis decreases after re-endothelialisation but does not disappear. The sound endothelium of the valves is naturally resistant to bacterial adhesion,[13] however, it is not clear why re-endothelialised valves are not. Plasma protein-mediated bacterial attachment might be altered on decellularised homograft tissue.

To address these questions, a newly designed low-volume flow chamber has been designed.

In the present study, we investigated in vitro bacterial adhesion to decellularised fresh homograft tissue and compared it to cryopreserved homograft and bovine pericardial patch tissue. Also, a potential contribution of plasma fibrinogen to the interaction between *S. aureus* and the tissue grafts was addressed.

Materials and methods

In vitro laboratory model to study cardiac tissues

To characterise the propensity of cardiac graft tissues for bacterial attachment, we have developed a new in vitro model of a parallel plate flow chamber (Fig 1). Pieces of the investigated cardiac graft tissues were mounted into this system presented in Fig 1 and submitted to the perfusion of bacterial suspensions at room temperature (details of all used methods are described in the supplementary material).

Graft tissues and bacterial adhesion

Bacterial adhesion and fibrinogen binding were tested for bovine pericardium patch (Supple Peri-Guard®; Synovis Surgical Innovations, St Paul, Minnesota, United States of America), cryopreserved pulmonary homograft (human origin, European Homograft Bank, Brussels, Belgium) and decellularised fresh pulmonary homograft (human origin, Corlife oHG, Hannover, Germany) under shear stress and static conditions, respectively. After overnight incubation with human pooled plasma to provide the fibrinogen binding, graft tissues were mounted into the parallel plate flow chamber and submitted to perfusion.

Bacterial suspensions of the *S. aureus* strain 8325–4 (suspensions of 10^7 colony-forming units/mL) were perfused over graft tissues. After serial dilutions, colony-forming units were counted.

Fibrinogen binding to graft tissues

Prepared graft tissues were incubated at 37 °C for 2 hours with fluorescently labelled human fibrinogen. After washing steps, the absolute fluorescence was quantified to express the degree of fibrinogen binding.

Statistical analysis

Non-parametric tests were used to analyse the results as the statistical evaluation revealed that not all data followed a Gaussian distribution. The t-test with the non-parametric Mann–Whitney U test was used to test a statistical significance between groups. Data analyses were performed with GraphPad Prism 8.0 d (GraphPad Software, San Diego, California, United States of America). Values are demonstrated as median and distribution plots using 25–75% percentile boxes with respective min and max binding whiskers. P-values <0.05 were considered significant.

Results

Cryopreserved and decellularised pulmonary homografts display a different propensity for S. aureus attachment in vitro

In the present study, we focused on in vitro bacterial adhesion to decellularised pulmonary homograft and compared it to cryopreserved homograft and bovine pericardial patch tissue under shear conditions generated in our laboratory model (Fig 1). We noted that bacterial adhesion to cryopreserved homograft was nearly 6-fold lower than to bovine pericardial patch. Interestingly, decellularised pulmonary homograft displayed a significant increase of bacterial adhesion by about 70% compared to the cryopreserved counterpart (based on median values), which was however

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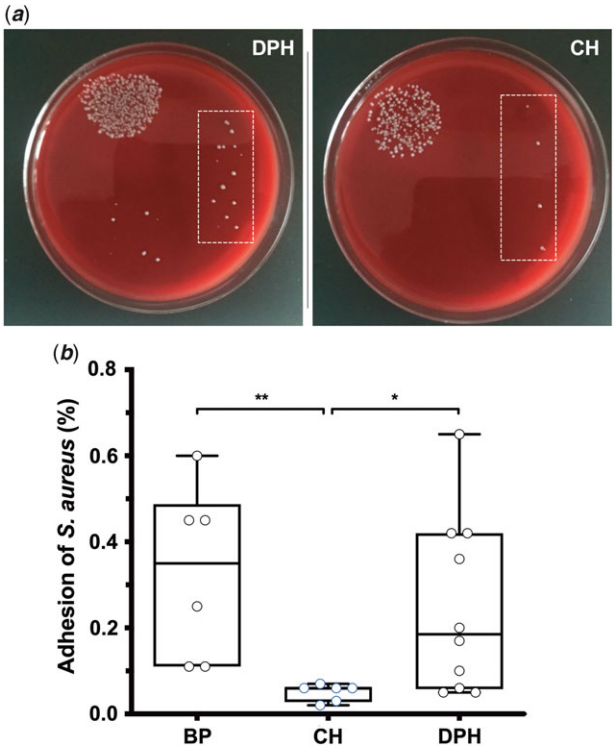

Figure 2. Adhesion of *Staphylococcus aureus* to cardiovascular grafts under shear stress. Perfusion of *S. aureus* over bovine pericardium patch (BP), cryopreserved pulmonary homograft (CH) and decellularised pulmonary homograft (DPH) ($n \geq 6$). (***a***) Representative Mueller-Hinton blood agar plates with *S. aureus* colonies, obtained as colony-forming units (CFUs) that bound, respectively, to DPH and CH; dotted squares indicate the same dilution. (***b***) Degree of bacterial adhesion to BP, CH and DPH. Results are presented as box and whisker dot plots with the upper and lower borders of the box representing the 25 and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median, the upper and lower whiskers the maximum and minimum values of non-outliers. T-test with non-parametric Mann–Whitney U test was applied: *$p < 0.05$, **$p < 0.01$.

characterised by a widespread (Fig 2 b). Values for the decellularised pulmonary homograft and bovine pericardial patch were not significantly different.

Figure 2 a shows *S. aureus* colonies on blood agar plates that bound, respectively, to decellularised pulmonary homograft (left image) and cryopreserved homograft (right image). Dotted squares indicate less colonies obtained for the latter tissue using the same dilution.

Plasma fibrinogen can influence S. aureus attachment to decellularised pulmonary homograft

It is known that plasma fibrinogen is an important mediator of bacterial adhesion to endothelial cells and to the subendothelial matrix. We have recently shown that plasma fibrinogen is able to solely support *S. aureus* adhesion to plasma-coated bovine tissues.[9] Therefore, we next aimed to investigate the degree of protein binding to the homograft tissues and evaluate its role in bacterial adhesion. We questioned now if decellularised homografts share similar properties with the cryopreserved counterparts in terms of fibrinogen binding. The fluorescently labelled protein was used in the binding assay, which showed that the bovine pericardial patch had a significantly higher affinity for fibrinogen compared to cryopreserved homograft ($p < 0.05$) (Fig 3 a). Interestingly, fibrinogen bound also more avidly to decellularised than to cryopreserved homograft or bovine pericardial patch ($p < 0.01$) (Fig 3 a). Based on this observation, plots combining *S. aureus* adhesion and fibrinogen binding for the three investigated tissues show that decellularised pulmonary homograft and cryopreserved homograft represent separate groups (Fig 3 b). The first "group" with the cryopreserved tissue presents hardly any affinity for both fibrinogen and *S. aureus*. The second "group" (decellularised pulmonary homograft and bovine pericardial patch) shows a higher degree of fibrinogen binding and bacterial adhesion. This finding may implicate fibrinogen as a molecule contributing to the bacterial recruitment to the surface of the tissue.

Discussion

Infective endocarditis remains a severe complication after valve replacement interventions despite advanced treatment options.[13,15] The prevalence of this infectious disease is rapidly increasing as a consequence of medico-surgical progresses and population ageing.[13] Although the underlying mechanisms for the different susceptibility of various heart valve tissues for infection remain unclear, some potential factors have been elucidated in vitro.[13,16] In this study, we addressed the potential role of the plasma protein fibrinogen in mediating *S. aureus* adhesion to decellularised tissues using a newly constructed flow chamber. Our results reveal that this plasma protein binds well to the decellularised homograft tissue and subsequently contributes to the process of *S. aureus* adhesion to the graft surface. Interestingly, this propensity of decellularised tissue for fibrinogen binding was significantly higher than that of cryopreserved homograft and bovine pericardial tissues. Fibrinogen plays a crucial role in platelet and *S. aureus* interaction since as a bridging molecule, it facilitates bacterial recruitment to the tissue surface via the activated platelet receptor $\alpha_{IIb}\,\beta_3$.[9,17]

As a consequence, our data show a relatively higher *S. aureus* adhesion to decellularised homograft and bovine pericardial tissues than to cryopreserved homograft in vitro. Herewith, we could highlight that the mechanism of bacterial adhesion via fibrinogen plays an important role, also for decellularised biomaterials such as decellularised homografts. In detail, we noted that bacterial adhesion to cryopreserved homograft was nearly 6-fold lower than to bovine pericardium, which supports earlier findings.[9]

Previous studies showed that differences in bacterial adherence to the bovine jugular vein and cryopreserved homograft tissues cannot be attributed to intrinsic tissue specificities,[18,19] as the tissue surface itself and the bacterial adhesins (surface molecules) do not seem to have a significant impact on the infection.[18] These findings triggered to investigate in detail the role of one of the abundant plasma proteins such as fibrinogen. The exact mechanisms of fibrinogen adsorption to the cardiac tissues are not fully known yet and awaiting further investigation, albeit some important properties like surface chemistry and topography have been elucidated.[10,20] Decellularisation of biological surfaces can be performed using various protocols,[21–23] leading to different physical and chemical properties of tissue matrices. Thereby, it becomes obvious that all types of surface manipulations of bioprosthetic grafts can influence the biological responses to their surfaces such as the protein adsorption (e.g., fibrinogen), platelet adhesion or blood coagulation.[10] Furthermore, commonly used tissue fixatives, like glutaraldehyde to ensure the tissue integrity, may also contribute to altered binding effects. Fibrinogen amongst others acts as a modulating protein for platelet adhesion and clustering by bridging their activated receptor $\alpha_{IIb}\,\beta_3$.[9,24] We developed the new flow

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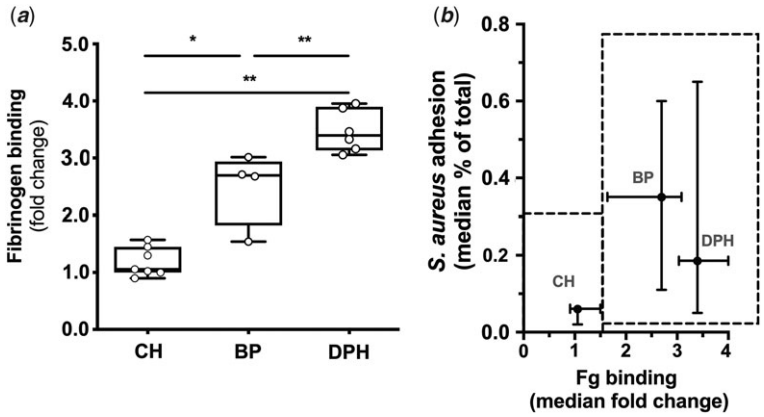

Figure 3. Binding of fibrinogen (Fg) to graft tissues in static conditions. (***a***) Cryopreserved pulmonary homograft, CH (n = 7), decellularised pulmonary homograft, DPH (n = 6), bovine pericardial patch, BP (n = 4) graft tissues were incubated with fluorescently labelled Fg (30 µg/mL). Values expressing Fg binding are presented as a fold change. Data are demonstrated as box and whisker dot plots with the upper and lower borders of the box representing the 25 and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median and the upper and lower whiskers the maximum and minimum values of non-outliers. t test with the non-parametric Mann–Whitney U test was applied: *$p < 0.05$, **$p < 0.01$. (***b***) Distribution of examined tissues in a high-affinity (BP and DPH) and low-affinity group (CH) is presented by dotted line boxes, based on a degree of their interaction with Fg and *S. aureus*, respectively, in static and flow conditions. Data are presented as dot plots, representing the median values with whiskers ranging from 25 to 75% percentile for both binding events, respectively.

chamber, presented in this study, to be able to investigate various tissues using only small amounts of perfusion media with bacteria. By this, the chamber allowed us to study the role of the most abundant plasma protein fibrinogen in bacterial adherence to cardiac graft tissues and will be of use to proceed with investigating the role of platelets in this process occurring on decellularised right ventricular outflow tract tissues. This chamber is superior to the previous design, which required a larger amount of perfusion medium in the circuit and a longer experimental procedure.[14]

Clinical reports highlight the risk to acquire infective endocarditis after right ventricular outflow tract valve replacement, which seems to be higher after implantation of heterologous bovine jugular vein conduits compared to cryopreserved homograft.[4,25] The recently introduced decellularised pulmonary homografts show a very good haemodynamical performance with a freedom of explanation of >96% which was similar or even higher to retrospective data after cryopreserved homograft and bovine jugular vein conduit implantation.[6,12] Importantly, the decellularised homografts are described with a low risk of associated infective endocarditis.[6,26] Follow-up studies report a low incidence of endocarditis with 96.2% of patients being free from the disease after 10 years, which is similar to the cryopreserved homograft with 97.4%.[6]

Looking more in detail into the differences between the "cell-free" homograft and cryopreserved homograft, after years of experience, it is postulated that the cryopreserved homograft seems to be more immunogenic than previously thought.[27] The main aim of the decellularisation process is to reduce the host immune reaction towards the tissue surface that inherits a great potential to become a more ideal graft. The presence of cellular antigens remaining on bovine jugular vein conduits and cryopreserved homografts may exacerbate inflammatory processes, explaining why potentially the decellularisation could reduce susceptibility for infective endocarditis.

Decellularised homograft tissues retain, however, the capacity for cellular repopulation as recipient endothelial cells are mainly covering the tissue over time.[23] Nevertheless, after implantation, the decellularised homograft tissues contain an exposed extracellular matrix, which might naturally allow for bacterial invasion and thereby facilitating the infiltration of immune-competent cells resulting in the activation of inflammatory cascades. Therefore, this leads to the recommendation of not using the decellularised homografts for valve replacement in case of active endocarditis.[28]

In conclusion, our results postulate that fibrinogen plays a role in bacterial recruitment to the surface of decellularised homograft tissue similarly as to the bovine graft tissues in vitro. Future work should essentially involve *in vivo* models to be able to characterise the role of other players such as platelets in the pathogenesis of infective endocarditis on various cardiac tissues, including the decellularised homografts.

Supplementary Material. To view supplementary material for this article, please visit https://doi.org/10.1017/S1047951120002772

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Conflicts of Interest. None.

Ethical Standards. The research does not involve human and/or animal experimentation.

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