Cell-mediated retraction versus hemodynamic loading – A delicate balance in tissue-engineered heart valves

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ABSTRACT

Preclinical studies of tissue-engineered heart valves (TEHVs) showed retraction of the heart valve leaflets as major failure of function mechanism. This retraction is caused by both passive and active cell stress and passive matrix stress. Cell-mediated retraction induces leaflet shortening that may be counteracted by the hemodynamic loading of the leaflets during diastole. To get insight into this stress balance, the amount and duration of stress generation in engineered heart valve tissue and the stress imposed by physiological hemodynamic loading are quantified via an experimental and a computational approach, respectively.

Stress generation by cells was measured using an earlier described in vitro model system, mimicking the culture process of TEHVs. The stress imposed by the blood pressure during diastole on a valve leaflet was determined using finite element modeling. Results show that for both pulmonary and systemic pressure, the stress imposed on the TEHV leaflets is comparable to the stress generated in the leaflets. As the stresses are of similar magnitude, it is likely that the imposed stress cannot counteract the generated stress, in particular when taking into account that hemodynamic loading is only imposed during diastole. This study provides a rational explanation for the retraction found in preclinical studies of TEHVs and represents an important step towards understanding the retraction process seen in TEHVs by a combined experimental and computational approach.

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1. Introduction

Heart valve dysfunction is a significant problem worldwide. Currently, 280,000 valve replacements are implanted every year worldwide (Pibarot and Dumesnil, 2009). This number is expected to increase up to 850,000 in 2050 (Yacoub and Takkenberg, 2005). Unfortunately, the inability to grow and remodel makes current valve replacements inadequate for pediatric applications (Zilla et al., 2008). This might be overcome by using tissue-engineered heart valves (TEHVs) (Yacoub and Takkenberg, 2005). Autologous TEHVs are fabricated by seeding extracellular matrix (ECM) producing cells onto a degradable synthetic scaffold. During culture inside a bioreactor system, the cells experience mechanical and chemical stimuli to induce the cells to produce ECM, while the scaffold slowly degrades.

In the last decades, significant progress in the development of TEHVs based on rapid degrading synthetic scaffolds has been achieved (Mol et al., 2009). Nevertheless, regurgitation is a persisting problem occurring in all preclinical studies with such valves (Weber et al., 2011; Flanagan et al., 2009; Gottlieb et al., 2010; Schmidt et al., 2010; Syedain et al., 2011; Dijkman et al., 2012). Regurgitation occurs due to cell-mediated retraction (shrinkage) of the TEHV leaflets, causing incomplete closure of the valve. In our group, TEHVs are cultured inside a bioreactor with the leaflets attached to each other, to assure constrained tissue culture (Mol et al., 2005a). Constrained tissue culture and the subsequent stress development appeared to be beneficial for in vitro tissue formation (Mol et al., 2005a) and alignment in engineered tissues (Neidert and Tranquillo, 2006; Robinson et al., 2008). Just before implantation, the leaflets are separated to allow valve opening and closure in vivo. Due to both active and passive cell stress generation, the tissue shrinks after removing its constraints. Thus, although cell traction is beneficial for matrix formation, it also causes tissue shrinkage.

Tissue shrinkage is hypothesized to result from an imbalance between the cell traction forces and the resistance of the newly formed ECM and scaffold. In the early phase of culture, the rapidly degrading synthetic scaffold is sufficiently stiff to withstand the traction forces of the cells. However, after approximately two weeks, the scaffold starts to degrade rapidly, while the neo-tissue is developing and may not yet have the capacity to withstand the...
cell traction forces (van Vlimmeren et al., 2011). These changes cause tissue shrinkage in two ways. First, during culture the cells remodel the tissue, causing compaction of the tissue in the non-constrained direction, which flattens the TEHV leaflets. Second, by separating the leaflets, retraction occurs immediately due to release of stress generated within the ECM by the cells during culture (van Vlimmeren et al., 2012; Balestrini and Billiar, 2009). This retraction slowly continues over time (Balestrini and Billiar, 2009) due to additional cell traction forces. It has been shown that 45% of the total tissue retraction is caused by active cell retraction, 40% by passive cell retraction, and 15% by passive retraction of the ECM (van Vlimmeren et al., 2012). This indicates that the role of cells in leaflet retraction is not only in an active manner, but also just by their presence.

When a TEHV is implanted, the stress that is imposed on the leaflets by the blood pressure during diastole might help to counteract the stress developed by the cells. Therefore, this might decrease or diminish the leaflet retraction. Leaflet retraction is reported in many animal studies in which TEHVs were implanted in pulmonary position (Dijkman et al., 2012; Flanagan et al., 2009; Gottlieb et al., 2010; Schmidt et al., 2010; Syedain et al., 2011). This may indicate that the stress developed in the leaflets was too high to be counteracted by the pulmonary blood pressure. However, little is known about the behavior of TEHVs in the systemic circulation, since only few short-term in vivo studies have been conducted (Emmert et al., 2011, 2012; Weber et al., 2011). Therefore, it is unclear if the systemic blood pressure, being approximately four times higher than the pulmonary pressure, would be sufficient to counteract the generated stress.

The aim of this study is to get insight into the balance of cell-mediated leaflet shortening and the stress imposed on the leaflets by physiological hemodynamic loading via an experimental and a computational approach, respectively. An earlier described in vitro model system containing engineered valvular tissues is used to quantify stress generation in the tissue. This mimics the separation of the leaflets and the hemodynamic load imposed on them directly after implantation.

2. Materials and methods

2.1. Experimental setup

van Vlimmeren et al. (2011) developed an in vitro model system, in which generated force, compaction, and retraction can quantitatively be measured during tissue culture and after release of constraints. The model system consists of a stainless steel frame with two ultra-high-molecular-weight polyethylene sliding blocks positioned opposite of each other (Fig. 1). A tissue-engineered (TE) construct can be placed between the two sliding blocks for culturing. The retraction-sliding block can either be fixed using a clamp (during culture) or move freely to release constraints. The other sliding block is attached to the frame via two leaf springs. The displacement of this block is related to the generated force in the tissue. This force, caused by the contraction of the ECM and cells, can be obtained by measuring the displacement of the sliding block. The displacement is measured by calculating the distance between a black dot on the sliding block and a reference dot on the stainless steel frame in pictures taken using a stereomicroscope (Discovery.V8; Zeiss, Sliedrecht, The Netherlands), which are analyzed using Matlab (the Math-Works, Natic, MA).

After four weeks of culture, the clamp of the fixed sliding block was released to allow movement of the block. After instant retraction (due to release of constraints) of the tissue occurred, the block was fixed again to enable force regeneration in the tissue.

2.2. Quantification of force and stress

In our experiments, the force (mN) generated by the TE construct was calculated using the displacement (mm) of the sliding block. All model systems were calibrated using an individual fit. Calibration was done by measuring the displacement of the sliding block during three cycles of loading to known forces ranging from 0 to 20 mN. Each individual fit was used to determine the force generated by the matching TE construct.

To translate the regenerated force to the Cauchy stress (kPa), the cross-sectional area (mm²) of the constructs was determined using histological sections. The TE constructs were fixed in 3.7% formaldehyde (Merck, Schiphol-Rijk, The Netherlands) in the model system. Constructs were released from the model system, processed, and embedded in paraffin. Tissue sections of 10 µm thick were cut and stained with Hematoxylin and Eosin. Stained sections were evaluated using bright field microscopy (Axio Observer; Zeiss). Images were analyzed using Matlab to calculate the cross-sectional area, assuming a linear shrinkage factor of 1.043 during processing of the tissue for histology (Schued et al., 1996).

2.3. Tissue culture

Human vena saphena cells (passage 7) were seeded onto rectangular scaffolds in a seeding density of 15 million cells per cm², using fibrin as a cell carrier (Mol et al., 2005b). The scaffold consists of rapid degrading nonwoven polyglycolic acid (Concordia Manufacturing Inc., Coventry, RI) coated with poly-4-hydroxybutyrate, obtained via collaboration with professor S.P. Hoorstrup (University Hospital Zürich, Zürich, Switzerland). The scaffold was attached at both ends to the sliding blocks using polyurethane-tetrahydrofuran glue (15% wt/vol). The TE constructs were sterilized by incubation in 70% ethanol for 30 min.

The TE constructs in the model systems were cultured in rectangular well plates for four weeks in a humidified atmosphere containing 5% CO₂ at 37 °C. Medium consisted of Advanced DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% Fetal Bovine Serum (Greiner Bio-One, Frickenhausen, Germany), 1% GlutaMax (Invitrogen), 1% penicillin/streptomycin (Lonza, Basel, Switzerland) and 0.25 mg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO) and was changed twice a week during culture.

2.4. Experimental design

Six TE constructs were cultured inside six model systems for four weeks and the experiment was done in triplicate (referred to as runs 1, 2, and 3 with n = 6 per run and n = 18 in total). After culture, the clamp holding the retraction-sliding block was removed and fixed again after instant retraction. Thereafter, force and stress development was measured for 77 h. Between the measurements, the TE constructs were kept at 37 °C and 5% CO₂. The generated force of the TE construct after

![Fig. 1. Schematic overview (a–b) and photographs (c–d) of the model system, in which force can be measured through displacement (v) of the sliding block. (1) Dots to measure generated force. (2) Clamp to fixate retraction-sliding block. (3) Dots to measure retraction. (4) Hole to hold the sliding block during fixation and release of the clamp. (c) Model system without the TE construct. (d) Model system with TE construct after four weeks of culture.](image-url)
four weeks of culture was used as a reference to calculate the relative force regeneration. Using the measured force regeneration, stress regeneration was calculated. For each of the three runs and for all three runs together, the experimental data were averaged. After averaging, the force and stress regeneration were fit with a biexponential fit using Matlab:

\[ y = a_1 (1 - 0.5e^{-t/h_1} - 0.5e^{-t/h_2}) \]  

where \( i \) represents force regeneration and \( s \) for stress regeneration, \( a_1 \) (%), represents the maximum amount of regenerated stress or relative regenerated force, and \( h_1, h_2 \) are time constants. All three fit parameters are measures for the amount and/or speed of force or stress generation. The higher \( h_1, h_2 \), the more force or stress is regenerated after release and subsequent fixation of constraints. The lower \( h_1, h_2 \), the faster the force or stress regenerates.

2.5. Finite Element Modeling

FE modeling was used to predict whether stress imposed on the heart valve leaflet during hemodynamic loading is large enough to counteract the stress regenerated by the cells. This TEHV model refers to the numerical model developed by Driessen et al. (2007). Briefly, the engineered tissue is modeled as an incompressible fiber-reinforced material. The FE mesh of the initial, stress-free closed configuration of the TE leaflets is shown in Fig. 2. Because of symmetry, only one half of one of the leaflets was modeled. The total Cauchy stress is split into a hydrostatic pressure (\( p \)) and an extra stress (\( \varepsilon \))

\[ \sigma = -p + \varepsilon \]  

The extra stress is split into an isotropic matrix part and an anisotropic fiber part respectively:

\[ \varepsilon = \varepsilon_s + \varepsilon_f \]  

where \( \varepsilon_s \) represents the matrix stress, \( \varepsilon_f \) the fiber volume fraction in the ith fiber direction after deformation, \( \varepsilon^i_f \) the fiber volume fraction is set to 0.5. The fiber stress, \( \varepsilon_f \), only acts in the fiber direction, \( \varepsilon^i_f \). The matrix is modeled as a Neo-Hookean material with a shear modulus, \( G \), of 50 kPa (Driessen et al., 2007). The anisotropic fiber stress is described by an exponential relationship between the fiber stress, \( \varepsilon_f \), and stretch, \( s_f \):

\[ \varepsilon_f = k_1 s_f^{0.5} \left( e^{(s_f - 1)} - 1 \right) \]  

where \( k_1 \) (kPa) is a stress-like parameter and \( s_f \) is a dimensionless parameter. It is assumed that the fibers cannot withstand compressive forces, therefore \( \varepsilon_f = 0 \) for \( s_f < 1 \). The relative number of fibers in each direction is distributed using a Gaussian function:

\[ \phi_f = A \exp \left( -\frac{(s_f - \mu)^2}{2\sigma^2} \right) \]  

with the main fiber direction (\( \mu \)) in the circumferential direction and a standard deviation (\( \sigma \)) which determines the amount of anisotropy in the leaflet.

The parameters \( k_1, k_2 \), and \( \sigma \) are set to 1689 kPa, 1.93, and 63.6 respectively, as described by Mol et al. (2006) for a four weeks cultured TEHV. Pulmonary pressure of 3 kPa (25 mmHg) and systemic pressure of 12 kPa (90 mmHg) were applied in the FE simulation. In both cases, radial and circumferential stresses were evaluated.

2.6. Statistics

All experimental data are presented as mean and the standard error of the mean. One-way ANOVA, followed by a Tukey’s multiple comparison post-hoc test, was carried out to evaluate statistical differences. Statistical analyses were done using SPSS Statistics 18 (SPSS Inc., Chicago, IL) and considered significant for \( p \)-values lower than 0.05. As the fits of the experimental data were made after averaging the data, no statistical analysis was performed on those data.

3. Results

3.1. Force and stress regeneration in engineered valvular tissues

After four weeks of culturing, force and stress regeneration was measured after release and subsequent fixation of constraints. For each of the three runs, the experimental data and fits described by Eq. (1) are shown in Fig. 3. The optimal fit parameters are summarized in Table 1. Force regenerated almost instantaneously in the first two hours (\( c_i \)), after which it slowly kept on increasing (\( b_i \)) towards 73% of the level of force just before release of constraints (Fig. 3a).

Similar to force, stress regenerated almost instantaneously in the first two hours (\( c_i \)), after which it slowly kept on increasing (\( b_i \)). After four weeks of culture, the stress reached 34 ± 4 kPa, 32 ± 5 kPa, and 23 ± 1 kPa for runs 1, 2, and 3 respectively. Immediately after release and subsequent fixation of constraints, the stress decreased to 11 ± 4 kPa, 6 ± 1 kPa, and 3 ± 2 kPa for the three runs (Fig. 3b). According to the fit parameters, the maximum regenerated stress (\( a_i \)) was 33 kPa, 18 kPa, and 16 kPa for runs 1, 2, and 3 respectively (Table 1). The overall maximum was 23 kPa, which is lower than the stress after four weeks of culture, before releasing the constraints. Although the experiments were performed using similar protocols, the measured force and stress of run 1 were significantly higher than that of run 2 between 8 and 26 h (\( p < 0.05 \), Fig. 3a). The force of run 1 was higher than that of run 3 at 24 and 26 h (\( p < 0.05 \), Fig. 3b). The stress of run 1 was higher than that of run 3 at 2, 8, 24, and 26 h (\( p < 0.05 \), Fig. 3b). After 26 h, no statistical comparison could be made since different time-points were used for each run. However, the force and stress of run 1 appear higher than that of run 2 and 3 at all time-points after 26 h (Fig. 3).

3.2. Stress imposed on TEHV leaflets by hemodynamic loading

FE simulation of a pressure-loaded heart valve model was performed to predict the level of stress imposed on TEHV leaflets by the pulmonary and systemic blood pressure. When pulmonary pressure was applied, the maximum principal stress ranged from −40 to 150 kPa (Fig. 4a and c). The highest stresses were found in the circumferential direction at the belly and commissure region, being over 50 kPa (Fig. 4a). The entire fixed edge and parts of the free edge of the leaflet showed negative stresses in the circumferential direction. Stress in the radial direction (Fig. 4c), was negative along the entire edge and only positive in the belly region. The maximum imposed stress in the radial direction was 18–20 kPa.

When systemic pressure was applied in the FE model, stress ranged from −50 to 250 kPa (Fig. 4b and d). The highest stresses, over 80 kPa (Fig. 4b), were found in the circumferential direction. In the circumferential direction, a small rim of negative stress was found at the fixed edge of the leaflets. In the radial direction, negative stresses were found in a small region along the entire leaflet edge with positive stress in the remainder of the leaflet (Fig. 4d). The maximum stress in the radial direction was 45–50 kPa in the belly region.

4. Discussion

In the present study, the amount and duration of the force and stress development in engineered valvular tissue were quantified
The experimental data of each run was averaged for all three runs together. Values shown are the parameters found when fitting with those of the three separate runs and for all three runs together. Values shown are the parameters found when the experimental data of each run was averaged first and the fit was made afterward.

### Table 1

Overview of the optimal values found for $a_i$ (%; maximum relative regenerated force), $b_i$ (h; time constant), $c_i$ (h; time constant), $a_i$ (kPa; maximum regenerated stress), $b_i$ (h; time constant), and $c_i$ (h; time constant) for each of the three separate runs and for all three runs together. Values shown are the parameters found when the experimental data of each run was averaged first and the fit was made afterward.

<table>
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<th>$a_i$ (%)</th>
<th>$b_i$ (h)</th>
<th>$c_i$ (h)</th>
<th>$a_i$ (kPa)</th>
<th>$b_i$ (h)</th>
<th>$c_i$ (h)</th>
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<td>63</td>
<td>0.08</td>
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<td>0.01</td>
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<td>61</td>
<td>0.03</td>
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<td>56</td>
<td>0.06</td>
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<td>67</td>
<td>83</td>
<td>0.02</td>
<td>16</td>
<td>85</td>
<td>0.07</td>
</tr>
<tr>
<td>Total</td>
<td>73</td>
<td>68</td>
<td>0.06</td>
<td>23</td>
<td>90</td>
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Experimentally and compared with FE analyses of the stress imposed on the TEHV by the physiological hemodynamic loading, this comparison provides insight into the in vivo retrogression process of TEHV leaflets that is observed in preclinical studies.

The model system used to quantify force and stress was developed previously to resemble the situation within a TEHV (van Vlimmeren et al., 2011). The valvular tissues in this model system are fabricated using seeding techniques, cells and scaffold material similar to those in TEHVs (Mol et al., 2005a). The tissue can compact during culture, resembling flattening of the leaflet during valve culture, due to traction forces exerted by the cells. After culturing, separation of the leaflets prior to implantation is simulated followed by fixation of constraints, mimicking the force imposed on the leaflets by the blood pressure during diastole directly after implantation.

Even though the experiments were run in triplicate in similar fashion, the experimentally measured force and stress of the first run were significantly higher than that of the other two runs. This might be caused by a difference in the quality of the formed extracellular matrix between each run, due to common variations in culture conditions, medium and scaffold batches. Therefore, this study emphasizes the need for multiple runs to draw solid conclusions.

The hemodynamic loading applied to the leaflets during diastole will cause them to extend. If this loading is large enough, the leaflet extension and the leaflet shrinkage by cell-mediated contraction may counterbalance each other. This would enable a stress equilibrium and shape conservation of the leaflet, causing less or no retraction. A similar mode of shape conservation is seen in the mitral valve, where the annulus tension and the myocardial force are in equilibrium (Bhattacharya and He, 2012). In the case of mitral valve prolapse however, the annulus tension decreases because of rupture or elongation of the chordae tendineae. This causes an imbalance between the mitral annulus tension and the myocardial force, resulting in insufficient closure of the valve (Bhattacharya and He, 2012).

A FE model developed by Driessen et al. (2007) was used to predict the amount of stress imposed on the TEHV by hemodynamic loading. Cell-mediated retraction in TEHVs is likely to occur mainly in the radial direction, as this is the least constrained direction. Therefore, a comparison of the experimentally determined regenerated stress to the numerically predicted stress imposed by hemodynamic loading in the radial direction is most relevant.

The maximum stress generated by the cells (23 kPa) is comparable with that imposed by the pulmonary blood pressure (18–20 kPa). As the stresses are of similar magnitude, it is likely that the imposed stress cannot counteract the generated stress. When systemic pressure was applied in the FE model, the maximum stress in radial direction was two times higher (45–50 kPa) than the maximum regenerated stress and might be better able to counteract cellular traction forces. Though, when taking into account that hemodynamic loading is only imposed during diastole and not in systole, regenerated and imposed stress may still be too similar to enable shape conservation.

When pulmonary pressure was applied in the FE model, negative stresses occurred along the edges in radial direction. This negative stress results in compression of the tissue during diastole and adds up to the stress regenerated by the cells causing increased leaflet retraction. When applying systemic pressure, this problem still occurred, although less severe. Overall, changes to retain shape of TEHVs are higher under systemic pressures as compared to pulmonary pressures.

Recent studies from our group showed that negative stresses do not occur in a computational model of a TEHV with improved anisotropy and geometry (Argento et al., 2012; Loerakker et al., 2013). In addition, these studies showed improved leaflet coaptation by increasing the radial stretches. These findings coincide with studies from other groups on the influence of anisotropy (Saleeb et al., 2013; Fan et al., 2013) and geometry (Fan et al., 2013) on the aortic valve closure and of mitral valve shape alterations on the valvular strain levels (Amini et al., 2012) and annulus tension levels (Bhattacharya and He, 2012). This indicates that improvements in the valve geometry and/or the amount of anisotropy represent promising approaches to decrease the amount of retraction of the TEHV leaflets in preclinical studies and to enable shape conservation for long-term functionality.

A limitation of the current study is that stress regeneration is examined under quasi-static conditions, while the heart valve leaflets are subjected to dynamic loads in vivo. Both the orientation...
and the contractile stress of myofibroblasts, as well as other valve-related cells, depend on the magnitude, frequency, and manner (uniaxial or biaxial stretch) of the cyclic deformation (Balachandran et al., 2011; Rubbens et al., 2009b; Rubbens et al., 2009a; van Geemen et al., 2013; Gould et al., 2012; Kaunas et al., 2006). Therefore, the overall tissue architecture and composition of the engineered valvular tissues described in this work and of the TEHV will not be completely similar. The cell stress found in this study is only achieved if all cells are aligned in the same direction (like in the engineered valvular tissues), while this is not the case in the more isotropic TEHV. This indicates that the maximum stress generated by the cells in a TEHV may probably be lower than that measured in this study. Though, it is clear that shape conservation will not occur under pulmonary conditions, systemic conditions may enable shape conservation to occur.

Fig. 4. Distribution of maximum principal stress (kPa) on the top surface of engineered heart valve leaflets after four weeks of culture imposed by pulmonary pressure of 3 kPa (a and c) and systemic pressure of 12 kPa (b and d) in the circumferential (a and b) and radial (d and d) direction. In (a) and (b), the scale bar ranges from −50 to 250 kPa, whereas (c) and (d) focus on the pressure between −60 and 60 kPa. At pulmonary pressure, stress ranges from −20 to 150 kPa in the circumferential direction with the negative stress only found on a few small spots on the edges. In the radial direction stress ranges from −40 to 20 kPa with positive values only found in the belly region of the leaflets. At systemic pressure, stress ranges from −50 to 250 kPa in the circumferential direction with negative stress only found on a few spots on the edges. In the radial direction stress ranges from −60 to 50 kPa with positive values only found in the belly region of the leaflets.

Incorporation of cell-mediated stress in the computational model is recommended for future studies. The experimentally determined cell stress can be incorporated into the FE model using recently developed cell models (Vernerey and Farsad, 2011; Obbink-Huizer et al., 2013; Deshpande et al., 2006). This will provide the opportunity of a more comprehensive analysis of the net stress distribution in the leaflets, in particular under cyclic loading conditions.

Taken together, this study represents an important step towards understanding the development of regurgitation by retraction in TEHV leaflets as observed in preclinical studies. A rational explanation of the observed retraction is the imbalance between the cell stress and hemodynamic loading of the valve, further enhanced by negative stress imposed during hemodynamic loading. Leaflet shape conservation is unlikely to occur under pulmonary conditions without adaptation of valve geometry and anisotropy. Under systemic conditions, there is a higher chance of shape conservation to occur.

Conflict of interest statement

The authors certify that they have no conflict of interest with respect to the material discussed in the manuscript.

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