Journal of Biomechanics ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

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

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ARTICLE INFO

Article history:
Accepted 31 October 2013

Keywords:
Heart valve tissue engineering
Stress generation
Retraction
Myofibroblast
Finite element method

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.

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 tissues 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

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0021-9290/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jbiomech.2013.10.049

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. The data obtained with this model system are compared to the results of finite element (FE) simulations of a TEHV model that analyses the amount of maximum principal stress imposed on the leaflets, under both systemic and pulmonary pressure. The results provide a first insight into the *in vivo* retraction process that is currently observed in TEHV in preclinical studies.

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 MathWorks, Natik, 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. This mimics the separation of the leaflets and the hemodynamic load imposed on them directly after implantation.

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 (mm^2) 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 (Schned 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 cm^3, using fibrin as a cell carrier (Mol et al., 2005b). The scaffold consists of rapid degrading nonwoven polyglycolic acid (Concordia Manufacturing Inc., Conventry, RI) coated with poly-4-hydroxybutyrate, obtained via collaboration with professor S.P. Hoerstrup (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% CO_2 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% CO_2. The generated force of the TE construct after

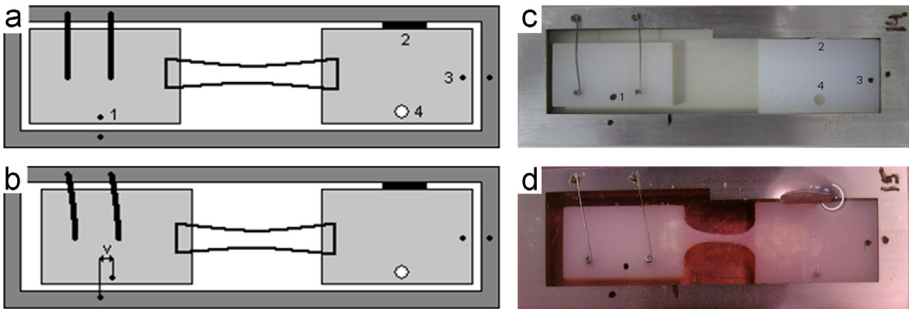

Fig. 1. Schematic overview (a–b) and photographs (c–d) of the model system, in which force can be measured through displacement (v) of the sliding block. (1) Dots to measure generated force. (2) Clamp to fixate retraction-sliding block. (3) Dots to measure retraction. (4) Hole to hold the sliding block 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.

Please cite this article as: van Loosdregt, I.A.E.W., et al., Cell-mediated retraction versus hemodynamic loading – A delicate balance in tissue-engineered heart valves. Journal of Biomechanics (2013), http://dx.doi.org/10.1016/j.jbiomech.2013.10.049