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Poly- ϵ -caprolactone scaffold and reduced *in vitro* cell culture: beneficial effect on compaction and improved valvular tissue formation

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Abstract

Tissue-engineered heart valves (TEHVs), based on polyglycolic acid (PGA) scaffolds coated with poly-4-hydroxybutyrate (P4HB), have shown promising *in vivo* results in terms of tissue formation. However, a major drawback of these TEHVs is compaction and retraction of the leaflets, causing regurgitation. To overcome this problem, the aim of this study was to investigate: (a) the use of the slowly degrading poly- ϵ -caprolactone (PCL) scaffold for prolonged mechanical integrity; and (b) the use of lower passage cells for enhanced tissue formation. Passage 3, 5 and 7 (P3, P5 and P7) human and ovine vascular-derived cells were seeded onto both PGA-P4HB and PCL scaffold strips. After 4 weeks of culture, compaction, tissue formation, mechanical properties and cell phenotypes were compared. TEHVs were cultured to observe retraction of the leaflets in the native-like geometry. After culture, tissues based on PGA-P4HB scaffold showed 50–60% compaction, while PCL-based tissues showed compaction of 0–10%. Tissue formation, stiffness and strength were increased with decreasing passage number; however, this did not influence compaction. Ovine PCL-based tissues did render less strong tissues compared to PGA-P4HB-based tissues. No differences in cell phenotype between the scaffold materials, species or cell passage numbers were observed. This study shows that PCL scaffolds may serve as alternative scaffold materials for human TEHVs with minimal compaction and without compromising tissue composition and properties, while further optimization of ovine TEHVs is needed. Reducing cell expansion time will result in faster generation of TEHVs, providing more rapid treatment for patients. Copyright © 2013 John Wiley & Sons, Ltd.

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Keywords heart valve; tissue engineering; scaffold; compaction; cell passage; extracellular matrix; mechanical properties

1. Introduction

With increasing number and ageing of the world population, valvular heart disease is an expanding health problem. Approximately 290 000 heart valve replacements are performed annually worldwide and this number is estimated to increase to 850 000 by 2050 (Yacoub and Takkenberg, 2005). Bioprosthetic and mechanical heart

valves, which have been successfully used for decades, improve quality of life and life prolongation for most patients (Mendelson and Schoen, 2006; Shahbudin, 2003). However, these valves have some restrictions, as they consist of non-living and non-autologous materials. Therefore, they are not able to grow, adapt or remodel to changing physiological environments, resulting in decreased durability (Yacoub and Takkenberg, 2005). Furthermore, bioprosthetic valves are susceptible to calcification, while mechanical valves require lifelong anticoagulation therapy to prevent thromboembolism (Mendelson and Schoen, 2006; Yacoub and Takkenberg, 2005). To overcome these problems, researchers are studying the

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possibility of creating tissue-engineered heart valves (TEHVs) (Mendelson and Schoen, 2006). Patients' own cells are incorporated, resulting in valves of autologous living tissue that are able to grow, remodel and adapt to the changing environment after implantation (Mendelson and Schoen, 2006). Our approach to create such TEHVs is to isolate patients' cells from the vena saphena magna, expand them *in vitro* to the desired amount of cells and subsequently seed them onto a biodegradable synthetic scaffold in the shape of a heart valve. After a culture period in a bioreactor of 4 weeks, where the valves are exposed to mechanical stimuli in order to stimulate tissue formation, the valves are able to withstand systemic pressures in *in vitro* tests (Mol *et al.*, 2006), aiming ultimately at implanting them into patients.

Different types of synthetic scaffolds are used for cardiovascular tissue-engineering applications. In particular, a polyglycolic acid (PGA) scaffold, coated with poly-4-hydroxybutyrate (P4HB) or combined with another scaffold material, was shown to be a promising candidate in terms of tissue formation, as demonstrated in vascular graft studies (Cummings *et al.*, 2011; Hoerstrup *et al.*, 2006) and *in vivo* TEHV studies (Gottlieb *et al.*, 2010; Hoerstrup *et al.*, 2000; Schmidt *et al.*, 2010; Weber *et al.*, 2011). Hoerstrup *et al.* (2000) demonstrated in an ovine model that after 20 weeks *in vivo*, the valves yielded an organized, layered structure with many architectural features and extracellular matrix characteristics that are present in native valves. *In vivo*, PGA and P4HB are degraded completely within 4 and 8 weeks, respectively. The down side of using this rapid degrading PGA scaffold is compaction (flattening of the leaflets) and retraction (shrinkage of the leaflets), causing regurgitation (Gottlieb *et al.*, 2010; Mol *et al.*, 2005a; Weber *et al.*, 2011). This is a result of traction forces exerted by the cells, likely in combination with an imbalance of the newly formed tissue and loss of mechanical integrity of the scaffold due to degradation (Mol *et al.*, 2006; van Vlimmeren *et al.*, 2011, 2012). Rabkin-Aikawa *et al.* (2004) demonstrated TEHVs containing α SMA-positive cells during *in vitro* culture, while after 20 weeks *in vivo* there was a strong decrease of α SMA-positive cells. As α SMA is related to traction forces of the cells (Chen *et al.*, 2007), we assume that after 20 weeks, these traction forces will be decreased *in vivo*. Therefore, a scaffold with proper mechanical integrity during *in vitro* culture and the first months after implantation is desired to withstand the cell traction forces during this phase. The use of a slower degrading scaffold material, such as poly- ϵ -caprolactone (PCL), may represent a promising alternative, as TEHVs can be produced that are mechanically reliable for months, thereby offering sufficient mechanical integrity to prevent tissue compaction and retraction (Klouda *et al.*, 2008). As PCL can be processed by electrospinning, it is possible to create complex geometries and mould the scaffold directly into the desired three-dimensional (3D) shape of a heart valve (Klouda *et al.*, 2008). This direct 3D moulding is not feasible for PGA scaffolds, which are only available in sheets. Another benefit of PCL is the possibility of creating thin leaflets

(thickness 300 μ m), while the PGA meshes are produced with a thickness of 1000 μ m. As PGA-P4HB scaffolds are more rapidly degrading, the cells might be exposed to larger magnitudes of mechanical loading than the cells in PCL scaffolds, which might on their turn be partly protected from loads by the long-term presence of the scaffold. As the stress level exerted on the vascular cells is known to change the phenotype of the cells towards activated myofibroblasts (Hinz, 2010), the tissue formation capacity of cells in the two scaffold types might differ, along with different phenotypes (Beamish *et al.*, 2010; Hinz *et al.*, 2007; Lacolley *et al.*, 2012; Rensen *et al.*, 2007). Therefore, it is important to compare cell phenotype, tissue formation capacity and compaction in tissues based on both scaffold types when considering the use of PCL as a scaffold material to produce TEHVs. Based on the above, we hypothesize that the cells in PGA-P4HB might have a more activated phenotype, accompanied by increased tissue formation capacity, than cells in PCL scaffolds.

Another alternative to tackling the compaction and retraction of TEHVs might be by using cells of a low passage number. Ageing cells, due to *in vitro* expansion, lose their potential to proliferate (Ragnauth *et al.*, 2010; Safwani *et al.*, 2012). Currently, in our laboratory, cells are expanded to passage 6–7 (P6–7) to ensure enough cells for seeding multiple TEHVs (Mol *et al.*, 2006). Whether the amount of tissue formation or cell phenotype in 3D cultures is influenced by the use of cells of a low passage number is still unclear, as to the best of our knowledge previous work on the effect of cell ageing by expansion has been performed on 2D cultures only. Therefore, the role of cell ageing in 3D tissue formation capacity needs to be investigated further. We hypothesize that cells of a low passage number (P3) are more productive, resulting in more tissue formation and of a higher quality, compared to cells of a high passage number (P7). This improved tissue-formation capacity in its turn may result in less compaction and retraction, as it is influencing the balance between matrix quality and the mechanical integrity of the scaffold towards increased matrix quality. We assume that the increased matrix formation will increase the resistance to the traction forces exerted by the cells. An additional benefit of using cells of a lower passage number is the reduction in cell expansion time, which will result in faster generation of TEHVs and, thereby, provide more rapid treatment to patients.

To summarize, the aim of this study was to evaluate alternative approaches to overcoming the compaction and retraction of TEHVs, as observed with the use of rapidly degrading PGA-P4HB scaffolds, without compromising tissue composition and properties. The alternative approaches studied here were: (a) the use of a slowly degrading PCL scaffold for prolonged mechanical integrity; and (b) the use of lower passage vascular cells for enhanced tissue formation. Compaction, tissue formation, cell phenotype and mechanical properties of engineered tissues based on P3, P5 and P7 vascular cells in both PCL and PGA-P4HB scaffolds were compared. TEHVs aim to be designed for humans, but since the

ovine model is commonly used to show proof of principle, both human and ovine cells were used.

2. Materials and methods

2.1. Cell culture

Human vascular-derived cells were harvested from segments of a vena saphena magna from a 60 year-old patient who underwent bypass surgery, and were obtained according to the Dutch guidelines for secondarily used materials. Ovine vascular-derived cells were obtained from the vena jugularis of adult sheep ca. 2 years old ($n = 2$; Swifter). The cells were isolated via the outgrowth method. In short, endothelial cells of the vessels were removed by incubation with a collagenase solution. The remaining endothelial cells were removed from the lumen side using a cell scraper. After removal of the endothelial cells, the vessels were minced into small pieces (ca. 1 mm²) and the fragments were plated into six-well plates. The outgrowing cells were expanded using standard culture methods in a humidified atmosphere containing 5% CO₂ at 37°C, and passaged at 90–100% confluency. Plating densities were $3.3\text{--}4.6 \times 10^3$ cells/cm² for human and $1.6\text{--}2.3 \times 10^4$ cells/cm² for ovine cells, based on differences in cell size. The isolation and expansion medium consisted of advanced Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Breda, The Netherlands), supplemented with 1% GlutaMax (Invitrogen), 1% penicillin–streptomycin (P-S; Lonza, Basel, Switzerland) and 10% fetal bovine serum (FBS; Greiner Bio One, Frickenhausen, Germany) for human cells or 10% lamb serum (Invitrogen) for ovine cells. During culture, cells of all passage numbers grew in the characteristic 'hill and valley' morphology, indicating smooth muscle cells.

2.2. Scaffold preparation and sterilization

Rectangular strips (25 × 5 mm) were cut out of PGA meshes (PGA, Cellon, Bascharage, Luxemburg) and conventionally electrospun PCL meshes, with thicknesses of 1000 and 300 µm, respectively. As heart valves contain a more complex geometry compared to strips, which might result in differences in terms of compaction, trileaflet heart valve scaffolds were fabricated using scaffold meshes of the same thickness. PGA scaffolds were additionally coated with poly-4-hydroxybutyrate (P4HB, received via a collaboration with Professor Hoerstrup of the University Hospital, Zurich) to provide structural integrity to the mesh. The outer 3–4 mm of both PGA and PCL scaffold strips were attached onto stainless steel rings (RVS Paleis, Geleen, The Netherlands) using 15% polyurethane–tetrahydrofuran (PU, Desmopan) glue, leaving an 18 × 5 mm area for cell seeding. The solvent was allowed to evaporate overnight in a vacuum oven. The PCL scaffolds were sterilized by γ -irradiation

(Isotron, Ede, The Netherlands). PGA–P4HB scaffold sterilization was achieved by immersion in 70% sterile ethanol for 30 min. To facilitate cell attachment, the scaffolds were incubated overnight with tissue-engineered (TE) medium, consisting of expansion medium supplemented with 0.25 mg/ml L-ascorbic acid 2-phosphate (Sigma). Lamb serum (0.1%) and FBS (10%) were added to ovine and human TE medium, respectively.

2.3. Cell seeding and tissue culture

P3, P5 and P7 cells were seeded onto both PGA and PCL scaffolds ($n = 6$ /passage and scaffold for each cell type) at a seeding density of 20 million cells/cm³, using fibrin as a cell carrier (Mol *et al.*, 2005b). In short, the cells were suspended in TE medium containing thrombin (10 U/ml, Sigma). This cell suspension was mixed with an equal volume of TE medium containing fibrinogen (10 mg/ml, Sigma) and dripped onto one side of the scaffolds before polymerization of the gel was accomplished. As control strips, three PGA and PCL scaffolds were seeded with fibrin only. After seeding, the scaffolds were placed in an incubator at 37°C for 30 min to allow polymerization of the fibrin gel. Thereafter, 6 ml TE medium was added to each scaffold. The strips were cultured for 4 weeks and the TE medium was changed twice a week. For the heart valve cultures, P7 cells were used and seeded according to similar protocols as for the strips. After seeding, the valves were placed in a bioreactor system and cultured for 4 weeks (Dijkman *et al.*, 2012b).

2.4. Compaction

Compaction was assessed from upper view photographs of the strips that were taken once a week. The valves were photographed after 4 weeks only. Compaction of the strips was defined as the reduction of width compared to the width at the start of culture. The photographs were analysed using the program ImageJ (v. 1.43u).

2.5. Biochemical assays

For the quantification of tissue formation after 4 weeks of culture, TE strips were lyophilized after mechanical testing ($n = 4\text{--}5$ /group) and used for biochemical assays. The total amount of DNA was determined as an indicator of cell number, the amount of hydroxyproline as an indicator for collagen content and the amount of sulphated glycosaminoglycans (sGAG). Measurements were averaged per group. Lyophilized tissue samples were weighed and digested in papain solution (100 mM phosphate buffer, pH 6.5, 5 mM L-cysteine, 5 mM ethylene-diamine-tetra-acetic acid (EDTA) and 125–140 µg papain/ml; all from Sigma) at 60°C for 16 h. After centrifuging the samples, the digest supernatant was collected and used for the DNA, sGAG and collagen assays. The amount of DNA in the TE strips was determined using the Hoechst dye method

(Cesarone *et al.*, 1979) and a standard curve prepared of calf thymus DNA (Sigma). Using the assumption that all cells contain 6.5 pg DNA (Dolezel *et al.*, 2003), the amount of cells per TE construct was calculated. sGAG content was determined using a modification of the protocol described by Farndale *et al.* (1986). In short, 40 μ l diluted sample was pipetted into a 96-well plate in duplicate, followed by the addition of 150 μ l/well dimethyl-methylene blue. Absorbance was measured at 540 nm. Subsequently, the amount of sGAGs in the TE strips was determined from a reference curve prepared from shark cartilage chondroitin sulphate (Sigma). Collagen content was determined by an assay as described by Huszar *et al.* (1980) and a standard curve was prepared from *trans*-4-hydroxyproline (Sigma).

2.6. Mechanical testing

After 4 weeks of culture, the mechanical properties of the TE strips ($n = 4$ –5/group) were assessed by uniaxial tensile tests in the longitudinal direction, with a uniaxial tensile stage (Kammrath and Weis, Dortmund, Germany) equipped with a 20 N load cell. Mechanical test data was averaged per group. Thickness of the strips was determined from representative histology sections. Samples were measured at three spots and mean thickness was used. The standard deviation (SD) of the measurements was in the range 1.5–10%. Stress–strain curves were obtained and, as a measure of tissue strength, the ultimate tensile strength (UTS) was defined as the peak stress value. The elasticity modulus (E-modulus) was determined as the slope of the linear (end) part of the curve, as a measure for tissue stiffness.

2.7. Histology

To analyse tissue formation qualitatively, TE strips were processed for histology ($n = 1$ /group). Representative tissue samples were embedded in tissue freezing medium (Tissue Tek, Sakura, Torrance, USA) and cryosections of 10 μ m were cut. The sections were formalin-fixed and studied by Masson's Trichrome (MT) staining (MTC kit, Sigma, Venlo, The Netherlands) for collagen deposition and by Picrosirius Red (PR) staining to assess the maturity of the collagen matrix (Junqueira *et al.*, 1979). The MT staining was analysed using light microscopy and the PR staining by polarized light microscopy (Axio Observer, Zeiss, Göttingen, Germany). In this study, maturity of the collagen fibres was assessed by the amount and density of the collagen fibres visible with polarized light microscopy. Mature fibres with a high density were coloured orange/red, while immature or less dense fibres were green.

Cell phenotype within the TE strips was analysed by immunofluorescence. After acetone fixation for 10 min, the sections were incubated with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature. After blocking, the sections

were incubated with a primary antibody overnight at 4°C. The antibodies used were mouse anti α -smooth muscle actin (α SMA) to stain smooth muscle cells and myofibroblasts (a2547, clone 1A4; 1:400; Sigma), mouse anti-smoothelin to stain contractile smooth muscle cells (clone R4A, 1:4; kindly provided by GJ van Eys, University of Maastricht) or rabbit anti-S100A4, which belongs to the S100 superfamily of cytoplasmic calcium-binding proteins, to stain fibroblasts and myofibroblasts (ab27957, 1:200; Abcam). After primary antibody incubation, the sections were washed with PBS and incubated with Alexa 488-labelled secondary antibodies (1:300; Sigma and Molecular Probes) to visualize the specific stainings and DAPI (1:500; Sigma) to stain all cell nuclei for 30 min at room temperature. After staining, the sections were mounted with Mowiol 4–88 (Calbiochem, San Diego, CA, USA) and visualized by fluorescent microscopy (Axiovert 200M, Zeiss, Göttingen, Germany).

2.8. Statistical analyses

All data are presented as mean $\pm$ standard error of the mean (SEM). Data of all experiments were normalized to human P3 PGA–P4HB strips in each experiment, in order to be able to compare experiments and perform statistical analyses. Pearson correlation coefficients were calculated to determine correlations between tissue parameters and cell passage numbers for both species and scaffold groups. Unpaired *t*-tests were used to compare the tissue properties between the scaffold materials within one cell passage and species, and to compare the tissue properties between species, within the same scaffold material and cell passage number. Statistics were performed using Graphpad Prism v. 5.04 and differences were considered significant at $p < 0.05$.

3. Results

3.1. Compaction after 4 weeks

The remaining width of the strips of all groups after 4 weeks of culture is shown in Figure 1A. A remaining width of strips of 100% is the initial width of the strips and represents no compaction. Tissues based on PCL scaffold and PCL and PGA–P4HB control strips showed compaction of 0–10%. Tissues based on PGA–P4HB scaffold resulted in significantly more compaction (ca. 50%) after 4 weeks ($p < 0.001$).

In ovine strips, no significant correlations were found between passage number and both types of scaffold. A negative correlation was found between human cell passage numbers and PGA–P4HB strips ($p < 0.01$), while there was a positive correlation between the human cell passage numbers and PCL strips ($p < 0.05$). This indicates that passage number and species did not consistently influence compaction. TEHVs based on PGA–P4HB scaffolds showed severe compaction and retraction of

Figure 1. (A) Compaction of strips after 4 weeks of culture. Initial width of strips was set at 100% (dotted line). PGA-P4HB showed around 50% compaction of the strips, while the use of PCL strips demonstrated reduced compaction, as the final reduction in width was only 0–10%. **Difference between the scaffold materials, $p < 0.001$; *, ## significant differences of $p < 0.05$ and $p < 0.001$, respectively, compared to human tissues. Negative or positive Pearson r correlations between the cell passage numbers are presented by arrows, combined with their p -values. Species and cell passage number did not consistently influence compaction of the TE strips. (B–E) Top-view photos of human PGA-P4HB (B); human PCL (C); ovine PGA-P4HB (D); and ovine PCL (E) TEHVs after 4 weeks of culture. Valves based on PGA-P4HB scaffold resulted in severe retraction of the leaflets after 4 weeks, while PCL valves did not show this. These results were consistent for both human and ovine cells

the leaflets after 4 weeks of culture in both species, while no compaction or retraction was observed in the PCL-based valves (Figure 1B–E), confirming the results found using the engineered strips.

3.2. Biochemical assays

Normalized collagen and sGAG per DNA of all groups are presented in Figure 2. Significant negative correlations between cell passage numbers and collagen amount per DNA were found in both human and ovine tissues of both scaffold materials ($p < 0.001$), demonstrating that increasing passage number resulted in decreased collagen per DNA. Low amounts of collagen per DNA were detectable in ovine PCL p7 strips. In general, ovine tissue strips demonstrated an increased amount of collagen when compared to human ($p < 0.001$). Collagen content per DNA of both human and ovine P7 cells seeded on PCL scaffolds was decreased, compared to human and ovine cells that were seeded on PGA-P4HB scaffolds ($p < 0.05$ for human cells; $p < 0.001$ for ovine cells). Although we showed that collagen and sGAG per DNA were increased with decreasing passage number, no differences in compaction of the tissues could be observed.

Biochemical parameters are related, as observed by correlation matrices, showing that collagen per DNA was increased when sGAG per DNA was increased. Overall, the amount of sGAG per DNA decreased with increasing cell passage ($p < 0.05$ for ovine PGA-P4HB strips; $p < 0.001$ for human PCL strips), although this effect was less pronounced with collagen per DNA. Except for ovine P7 PCL strips, ovine cells resulted in a higher amount of sGAG per DNA compared to human cells ($p < 0.05$ for ovine p3 PCL strips; $p < 0.001$ for all other ovine strips). No consistent differences in sGAG content by the cells were observed due to different scaffold materials.

3.3. Mechanical testing

Figures 3A and 3B, show averaged stress–strain curves of human and ovine P3 strips, which are representative for the other passage numbers, and the PGA-P4HB and PCL control strips. Bare PCL strips were able to bear higher stresses than bare PGA-P4HB strips, which was due to the differences in degradation time of the two scaffold materials. The PGA-P4HB cultured tissues of both human and ovine cells showed typical non-linear mechanical behaviour representing tissue behaviour. When PCL

Figure 2. Collagen per DNA (A) and sGAG per DNA (B). #, ## Significant differences compared to human tissues; ***significances of differences between scaffold materials, $p < 0.05$ and $p < 0.001$, respectively. Pearson r correlations between the cell passage numbers are presented by arrows combined with their p -values. Collagen per DNA decreases with increasing passage number in both human and ovine tissues and both scaffold materials (A). sGAG per DNA show the same trends, although less distinct (B). The scaffold does not influence the amount of collagen and sGAG formed, while more collagen and sGAG per DNA are formed within ovine tissues compared to human tissues

Figure 3. Mechanical data of engineered strips. Averaged stress-strain curves of human (A) and ovine (B) p3 strips are given as mean $\pm$ SEM. PGA-P4HB-based tissues demonstrate non-linear curves in both human and ovine strips, representing tissue behaviour. The stress-strain curve of human PCL strips is linear, while ovine strips follow the curve of the control scaffolds. Control PCL scaffolds are still influencing mechanical properties after 4 weeks of culture, while PGA-P4HB scaffolds are not. Tissue stiffness (C) and strength (D) increase with decreasing passage number. #, ## Significant differences compared to human tissues; ***significances of differences between scaffold materials, $p < 0.05$ and $p < 0.001$, respectively. Pearson r correlations between the cell passage numbers are presented by arrows combined with their p -values. In human samples, the highest values are obtained in PCL strips, while in ovine samples this is observed in PGA-P4HB scaffold strips

scaffold was used, the human tissues showed linear mechanical behaviour, while the ovine tissues were following the curve of the control PCL strips. Thus, PCL scaffolds are still influencing the mechanical properties of the engineered tissues after 4 weeks of culture, while PGA-P4HB scaffolds do not.

With a decrease in cell passage numbers, the parameters stiffness and strength were increasing in both species and scaffold materials, as significant negative correlations were observed between increasing cell passage numbers and both the stiffness ($p < 0.05$ for human PGA-P4HB strips; $p < 0.001$ for human PCL and ovine PGA-P4HB strips) and strength ($p < 0.05$ for human PGA-P4HB and

ovine PCL strips; $p < 0.001$ for human PCL and ovine PGA-P4HB strips) in human and ovine tissues based on both scaffold materials (Figure 3C, D). In human tissue samples, stiffness was higher in PCL samples compared to PGA-P4HB samples ($p < 0.05$ in P3 and P7 tissues), while in ovine tissue samples a higher stiffness was obtained in tissues based on PGA-P4HB scaffolds compared to PCL scaffolds ($p < 0.05$). Furthermore, tissue strength was increased in human PCL samples of all passage numbers and ovine PCL samples at P5 and P7 compared to PGA-P4HB tissue samples ($p < 0.05$), which was probably due to the influence of the PCL scaffold not yet degraded. When PCL scaffold was used,

the values of the mechanical properties of the ovine tissues were equally or just slightly increased compared to the PCL control strips, while the values of the human tissues were higher than the control strips (data not

shown). This indicated that tissues newly formed by ovine cells were not of the same quality as their human counterparts, as the added value of tissue to the mechanical properties of the ovine strips was relatively low.

Figure 4. Picosirius Red-stained sections of human PGA-P4HB (A–C), PCL (D–F), ovine PGA-P4HB (G–I) and PCL (J–L) visualized by polarized light microscopy. Maturity of collagen fibres is visualized as green (immature) and orange/red (mature). Most red fibres are visualized in tissues based on cells with a low passage number, indicating that maturity of collagen fibres after 4 weeks of culture is decreasing with increasing passage number. White scale bars = 200 μ m. Vacuoles within the scaffolds are cutting artifacts due to scaffolds remnants, and grey parts in the PGA-P4HB groups are P4HB remnants

Correlation matrices demonstrated that the mechanical parameters were related to one another, resulting in increased tissue strength when tissue stiffness obtained higher values, while the mechanical parameters were not related to the matrix properties of the tissues.

3.4. Histology

Histology of the TE strips revealed cellular tissue with dense surface layers. Picrosirius Red and Masson's Trichrome stainings (Figures 4, 5) showed collagen fibres in strips of all groups after 4 weeks of culture. A higher number of red fibres was seen in most tissues with cells of a low passage number (Figure 4). This indicated that tissues based on a low cell passage number resulted in more

mature collagen fibre formation. Histology furthermore indicated that the total amount of collagen fibres decreased with increasing passage numbers in both PGA-P4HB and PCL strips (Figure 5). Ovine PGA-P4HB tissues showed a higher amount of collagen compared to the human tissues. However, ovine PCL-based tissues showed smaller amounts of collagen than human PCL-based tissues. The total amount of collagen was higher in PGA-P4HB strips than PCL strips, which can be explained by triple the amount of cells seeded onto the PGA-P4HB strips compared to the PCL strips, due to differences in thickness of the scaffold materials.

Immunofluorescent stainings indicated no differences in cell phenotype with cell passage number, scaffold material or species, as tissues of all groups contained cells that were α SMA- and S100A4-positive and smoothelin-negative (Figure 6), indicative of synthetic myofibroblasts.

Figure 5. Masson's Trichrome staining of human PGA-P4HB (A–C), PCL (D–F), ovine PGA-P4HB (G–I) and PCL (J–L) sections. Blue scale bars = 200 μ m. Collagen is shown in blue and cytoplasm and muscle tissue in red. Vacuoles within the scaffolds are cutting artifacts due to scaffolds remnants. The total amount of collagen fibres seem to decrease with increasing passage number in both scaffold materials. Ovine PGA-P4HB strips show more collagen than human strips, while in PCL strips most collagen is visualized in human samples

Figure 6. Representative photomicrographs of immunofluorescent stainings of α SMA (A), S100A4 (B) and smoothelin (C) cell markers; white scale bars = 200 μ m. Protein of interest is shown in green; DAPI is visible in blue-stained cell nuclei. All stained tissues contain cells that were α SMA- and S100A4-positive and smoothelin-negative. This indicates that passage number, scaffold material and species are not influencing cell phenotypes. Vacuoles within the scaffolds are cutting artifacts due to scaffolds remnants

Cells in all strips were distributed homogeneously throughout the strips, as shown by cell nuclear staining (DAPI).

4. Discussion

Compaction and retraction of heart valve leaflets *in vitro*, resulting in regurgitation *in vivo*, is a common problem in TEHV that are based on rapidly degrading PGA-P4HB scaffolds. Therefore, alternative approaches to overcome compaction and retraction of TEHV are needed to meet *in vivo* demands. This study focused on the effect of two alternative approaches: (a) the use of a slowly degrading PCL scaffold; and (b) the use of lower-passage vascular cells. Compaction, tissue formation, cell phenotype and mechanical properties of both human and ovine tissues were investigated.

4.1. Differences due to vascular cell expansion times

In this study, we demonstrated that reduced *in vitro* expansion time of vascular cells resulted in improved tissue amount, as sGAG per DNA, collagen per DNA, tissue strength and stiffness were increased with decreasing passage number. A comparison of the net amounts of collagen and sGAG could not be made, as different amounts of cells were seeded, due to differences in scaffold thickness. Therefore, collagen and sGAG were normalized to DNA. A two-dimensional (2D) study of ovine jugular vein-derived cells showed that sGAG content was highest in low-passage cells (Hoffman-Kim *et al.*, 2005). Although cells in 2D may act differently compared to cells in 3D, our data also indicated that cells with an increasing passage number became less synthetic, as collagen and sGAG contents were decreased by cells of a higher passage number. Some *in vitro* studies showed that the vascular contractile smooth muscle cell marker smoothelin disappeared within a few days of *in vitro* expansion, and cells differentiated into synthetic, tissue-producing cells (van der Loop *et al.*, 1996), while others

observed this only after P9–P11 (Christen *et al.*, 1999; Teng *et al.*, 2006). All our human and ovine cells were differentiated into the synthetic phenotype, as no change of phenotype could be observed in this study due to cell passage number, and all tissue sections were shown to be α SMA- and S100A4-positive and smoothelin-negative, indicating activated, synthetic myofibroblasts. The cell phenotype of our samples and the amount of tissue were not related, as no change in cell phenotype could be observed, while it was shown that the amount of tissue increased with decreasing passage numbers.

The tissue stiffness of strips was obtained from the linear end part of the stress–strain curves and represents the end stiffness of our tissues. Increase of tissue stiffness was seen in strips based on a decreased cell passage number. The increase in end stiffness of our tissues resulted in stronger tissues, although native leaflets still show much higher values of stiffness than our tissues, 15.6 ± 6.4 MPa in the circumferential direction and 2.0 ± 1.5 MPa in the radial direction (Balguid *et al.*, 2007). Native valves are also more flexible than our engineered strips when comparing the physiologically relevant stiffness. The opening and closing functions of the heart valves are controlled by pressure differences. As the native valves are more flexible than their engineered counterparts, a lower pressure is needed for opening the valves.

Histology of the PGA-P4HB samples confirmed the biochemical results of collagen content per DNA, as higher amounts of collagen were observed in the ovine PGA-P4HB tissues compared to the human tissues. This increased amount of collagen in ovine PGA-P4HB-based tissues is not only explained by increased synthetic ovine cells but also by a higher proliferation rate of these cells compared to human cells when seeded on PGA-P4HB scaffolds (proliferation data not shown). However, ovine PCL-based tissues show little collagen in the histology slides compared to human PCL-based tissues, while the biochemical data showed an increased amount of collagen per DNA in ovine tissue compared to human. This can be explained by the proliferation rate of ovine and human cells in PCL scaffolds. As human cells showed a higher proliferation rate when seeded onto PCL scaffolds (data not shown) and, therefore, an increased amount of

total DNA per strip in PCL scaffolds compared to ovine cells, the amount of collagen per DNA is lower in human, while the total amount of collagen per strip might be higher, due to the presence of more collagen-producing cells. More research is needed to investigate why differences in proliferation rates of ovine and human cells are present when different types of scaffolds are used.

Mechanical results also correlated with the histological findings. Strips that showed more, and increased maturity of, collagen fibres also resulted in an increased tissue stiffness and strength. This is in line with previous findings, where a dominant role for collagen maturity by cross-linking of the collagen over collagen content was found with respect to the mechanical properties of the tissues (Balguid *et al.*, 2007).

It is remarkable that ovine p7 PCL strips resulted in only a few cells being present after 4 weeks. The collagen content of these cells was also low, resulting in weak strips, as observed in the tensile tests. We hypothesize that this might be due to the combination of several factors. One might be the use of a low amount of serum (0.1% in ovine 3D medium). This could have resulted in non-synthetic and non-dividing cells. In combination with the high passage number, which also showed to result in less activated or synthetic cells, this could have been the reason for the low amount of cells present after 4 weeks and the reduced amount of collagen. Furthermore, the use of PCL scaffolds is likely to have influenced the amount of collagen, as ovine P7 cells seeded on PGA-P4HB scaffolds did show higher amounts of collagen. We hypothesized that the use of PCL scaffold with ovine cells resulted in non-synthetic cells, as the mechanical integrity of this scaffold was present for a longer time span, resulting in no urgent need for the cells to create tissue. However, culturing TEHVs with ovine P7 cells did result in proper tissue formation. This might be explained by the different culture protocols of engineered strips and TEHVs. TEHVs undergo mechanical loading in a bioreactor during culture, while strips are cultured statically. Furthermore, interspecies differences might have played a role, as cells of a different sheep were used to culture the TEHVs.

Concerns might arise about the clinical applicability of using cells with a low passage number, mainly in children, as a relatively large number of cells need to be obtained. However, in the case of children, fewer cells are needed to be able to produce a TEHV compared to adults, as the annulus of the pulmonary valve in children is 10–17 mm, while this is around 25 mm in adults. The size of the leaflets in young patients is also smaller. Furthermore, when PCL-based TEHVs are produced instead of PGA-P4HB-based TEHVs, fewer cells are needed, due to differences in scaffold thickness. To produce a PCL-based TEHV scaffold for adults, 20×10^6 cells are needed, while this would be $2\text{--}10 \times 10^6$ cells in the case of children. These amounts of cells can be obtained by the outgrowth method, as the saphenous vein segments need to be only 1 cm. In conclusion, cells from a lower passage number were demonstrated to increase the amount of

tissue formation and tissue strength without influencing cell phenotype. Despite the improved tissue formation, compaction of the tissues was not influenced by a lower cell passage number.

4.2. PGA-P4HB vs PCL scaffold

In this study, we demonstrated that human and ovine tissues cultured for 4 weeks using PCL scaffold strips showed almost no compaction (0–10%), while PGA-P4HB-based tissues showed compaction up to 50%. Furthermore, we showed that PGA-P4HB-based TEHVs resulted in severe retraction of the leaflets in both species, while this was not seen in the PCL-based TEHVs. This demonstrates that PCL is a promising scaffold material to reduce compaction and retraction in TEHVs. Dijkman *et al.* (2012b) described another approach to prevent compaction and retraction of PGA-P4HB-based TEHVs. Trileaflet heart valves of PGA-P4HB scaffolds were seeded with ovine myofibroblasts and subsequently decellularized to prevent retraction. Decellularization represented to be a powerful tool to reduce tissue retraction, as it was shown that cell-induced retraction accounted for 85% of total tissue retraction. Residual matrix stresses are known to still account for 15% of the total retraction (van Vlimmeren *et al.*, 2012). These residual matrix stresses minimized the coaptation area in the study of Dijkman *et al.* (2012b) and it has to be elucidated in future studies whether this will influence *in vivo* valve behaviour. We believe that by using a slowly degrading scaffold, retraction can be even more effectively reduced by resisting residual matrix stresses while maintaining tissue viability.

Results of the mechanical tests demonstrated that in PCL strips the mechanical properties were not determined only by the formed tissue, but also by the remaining PCL scaffold, as it was not yet degraded. PGA-P4HB is known to start to degrade after 2 weeks, and therefore was not influencing the mechanical properties of the tissues. As amounts of sGAG and collagen per DNA were not influenced by the scaffold materials, the increased tissue strength of the human PCL strips compared to the PGA-P4HB strips are likely due to the remaining PCL scaffold. Ovine strips did not show the same results, which might be due to the low amount of DNA and, therefore, a lower amount of total tissue, in ovine PCL strips. The mechanical properties of the ovine PCL strips were mainly influenced by the remaining scaffold and not by the formed tissue, while in PGA-P4HB strips the measured mechanical properties represented tissue only. Furthermore, ovine tissues based on PCL scaffolds did not influence mechanical properties as much as human PCL tissues, as tissue strength and stiffness values were equally or just slightly increased compared to the PCL control strips. This indicated that the newly formed tissues based on ovine cells were not of the same quality as their human equivalents.

Differences in scaffold thickness could possibly have resulted in differences in tissue formation, due to variation

in nutrient and oxygen levels within the tissues. This is mainly seen in ovine strips, as human strips show more homogeneously distributed tissue. Our ovine strips possess a denser layer of collagen and cells on the surface in both scaffold materials. However, cells were not present only at the surface layer but also distributed throughout the centres of both scaffold materials. Not only the cells at the surface layer but also the cells in the centre produced collagen and expressed the synthetic smooth muscle cell markers, as visualized by histology. Furthermore, biochemical assays demonstrated no influence of the scaffold materials on the collagen and sGAG formation per DNA, and differences in mechanical properties of the tissues are most likely due to PCL scaffold remnants instead of differences between material thicknesses. Directly after seeding, the high porosity of the scaffold strips allowed oxygen and nutrient supply to the cells that were situated on the scaffold fibres in the middle part of the strip. When tissue was produced, porosity decreased and oxygen and nutrient supply might have been decreased, resulting in the formation of surface layers.

Native human heart valve leaflets are avascular, as they are thin enough to receive nutrients and oxygen through diffusion and haemodynamic convection (Butcher *et al.*, 2011). As PCL scaffolds are 300 μm , we do not expect problems when placing PCL TEHV *in vivo*. TEHVs based on PGA-P4HB did show increased thickness in the ovine model (Schmidt *et al.*, 2010), which might lead to reduced oxygen and nutrient supply to the cells in the centre. This problem might be less pronounced in humans, as these tissues are also compacting in the vertical direction, and therefore decreasing in thickness.

We conclude that the use of PCL scaffold seems to be an alternative scaffold material for the culture of human TEHVs to reduce compaction, while further optimization is needed when ovine cells are used, to ensure proper tissue formation.

4.3.. Interspecies differences

Tissue properties were different between species. In our study, ovine cells were shown to be more synthetic than human cells, as they contained more sGAG and collagen per DNA, while a study by van Geemen *et al.* (2012) demonstrated the opposite effect. Van Geemen *et al.* showed that human P7 cells contained double the amount of sGAG per DNA ($4.8 \pm 0.8 \mu\text{g}/\mu\text{g}$ DNA in ovine and $8.2 \pm 1.4 \mu\text{g}/\mu\text{g}$ DNA in human cells) and five times the amount of collagen per DNA ($1.1 \pm 0.3 \mu\text{g}/\mu\text{g}$ DNA in ovine and $5.9 \pm 2.5 \mu\text{g}/\mu\text{g}$ DNA in human cells) compared to ovine P7 cells. Tissues based on P7 cells in our experiments obtained values for sGAG per DNA of $6.5 \pm 0.2 \mu\text{g}/\mu\text{g}$ ovine DNA and $5.5 \pm 0.3 \mu\text{g}/\mu\text{g}$ human DNA. Collagen per DNA was $3.2 \pm 0.1 \mu\text{g}/\mu\text{g}$ DNA, and $3.7 \pm 0.3 \mu\text{g}/\mu\text{g}$ DNA in ovine and human, respectively. This suggests that ovine cells in our study were more synthetic or less proliferative, which might be due to the amount of serum used in the culture medium. Van Geemen used 2.5% lamb serum, while in this study only 0.1% serum

was used, as an *in vitro* TEHVs study by Dijkman *et al.* (2012a) demonstrated more homogeneous tissue formation throughout the wall and leaflets when 0.1% lamb serum was used. A review by Mol *et al.* (2009) described that the outcome of ovine TEHVs was dramatically different from their human equivalents when using the same culture conditions, and lower amounts of serum resulted in tissue outcome comparable to that of humans. This shows the difficulties in the translation step from animal studies towards the clinic and vice versa. Furthermore, previous studies showed that not only interspecies but also intraspecies variations of tissue properties are large (Balguid *et al.*, 2007; Kortsmits *et al.*, 2009; van Geemen *et al.*, 2012). Within this study we investigated the tissue properties of the strips seeded with cells of one sheep and one patient only. While it would be preferred to have more data on several human and ovine cell sources, we assume that within species the effects of, for example, cell passage number are comparable. Furthermore, the first goal of this study was to compare different types of scaffold to prevent compaction. This was investigated on cells of two species (human and ovine) and different cell passage numbers of those species. While two species and cell passage numbers were used and differences in terms of tissue production were observed between these species and cells passage numbers, the outcome of compaction was similar in all research groups. This indicates that the influence of the scaffold type is larger than the influence of the tissue production of several cell sources, in terms of compaction.

A limitation of our study is that the ovine cells originated from a young, healthy sheep, while the human vascular-derived cells were obtained from an older person who had undergone bypass surgery. This might have influenced the outcome of the tissue properties, as not only cell passage number but also patient age may have an effect on cell functioning, doubling time and ability of tissue production in different cell types (Han *et al.*, 2010; Klinger *et al.*, 2006; Ragnauth *et al.*, 2010).

In conclusion, differences in absolute values between ovine and human samples were seen within this experiment, although the general effects of reducing cell passage numbers and the use of PCL scaffold on compaction and the amount of tissue formation were comparable.

5. Conclusion

This study showed that PCL may serve as an alternative scaffold material for human TEHVs, with minimal compaction and without compromising tissue composition and properties, while further optimization of ovine TEHVs based on PCL scaffolds is needed, to ensure not only reduced compaction but also strong tissues of a high quality. Cells from lower passages demonstrated to improve tissue formation, without influencing compaction and cell phenotype. In addition, reducing cell expansion will result in faster generation of TEHVs, providing more rapid treatment to patients.

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Conflicts of interest

Anita Driessen-Mol and Frank P. T. Baaijens are shareholders of Xeltis.

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