Cardiovascular Engineering and Technology, Vol. 10, No. 4, December 2019 *(© 2019)* pp. 628–637
https://doi.org/10.1007/s13239-019-00438-x

Original Article

Non-physiologic Bioreactor Processing Conditions for Heart Valve Tissue Engineering

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(Received 24 June 2019; accepted 13 October 2019; published online 24 October 2019)

Associate Editor Jane Grande-Allen oversaw the review of this article.

Abstract
Purpose—Conventional methods of seeding decellularized heart valves for heart valve tissue engineering have led to inconsistent results in interstitial cellular repopulation, particularly of the distal valve leaflet, and notably distinct from documented re-endothelialization. The use of bioreactor conditioning mimicking physiologic parameters has been well explored but cellular infiltration remains challenging. Non-characteristic, non-physiologic conditioning parameters within a bioreactor, such as hypoxia and cyclic chamber pressure, may be used to increase the cellular infiltration leading to increased recellularization.
Methods—To investigate the effects of novel and perhaps non-intuitive bioreactor conditioning parameters, ovine aortic heart valves were seeded with mesenchymal stem cells and cultured in one of four environments: hypoxia and high cyclic pressures (120 mmHg), normoxia and high cyclic pressures, hypoxia and negative cyclic pressures (− 20 mmHg), and normoxia and negative cyclic pressures. Analysis included measurements of cellular density, cell phenotype, and biochemical concentrations.
Results—The results revealed that the bioreactor conditioning parameters influenced the degree of recellularization. Groups that implemented hypoxic conditioning exhibited increased cellular infiltration into the valve leaflet tissue compared to normoxic conditioning, while pressure conditioning did not have a significant effect of recellularization. Protein expression across all groups was similar, exhibiting a stem cell and valve interstitial cell phenotype. Biochemical analysis of the extracellular matrix was similar between all groups.
Conclusion—These results suggest the use of non-physiologic bioreactor conditioning parameters can increase *in vitro* recellularization of tissue engineered heart valve leaflets. Particularly, hypoxic culture was found to increase the cellular infiltration. Therefore, bioreactor conditioning of tissue engineered constructs need not always mimic physiologic conditions, and it is worth investigating novel or uncharacteristic culture conditions as they may benefit aspects of tissue culture.

Keywords—Tissue engineering, Heart valve, Bioreactor conditioning, Hypoxia.

INTRODUCTION

Heart valve tissue engineering is a promising solution for patients suffering from valvular heart disease and requiring valve replacement.[17] Unfortunately, the current options for valve replacement all suffer from limitations and the pediatric population is particularly disadvantaged by the lack of an ideal prosthetic valve substitute.[35] Mechanical prosthetic valves have poor hemodynamics requiring a lifetime of anti-coagulation therapy and tissue-based prosthetic valves are limited by poor durability often requiring re-intervention.[35] The tissue engineered heart valve (TEHV) has the potential to overcome existing limitations of current replacement options by providing a one-time implantation of living tissue capable of optimal hemodynamics, tissue remodeling, and growth.

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In creating a TEHV, decellularized heart valve conduits are a promising option to use as a construct scaffold. Decellularized valves, both allografts and xenografts, have been used as stand-alone clinical implants with poor results observed in decellularized xenografts from incomplete removal of xeno-antigens and superior results of decellularized allografts compared to their cryopreserved counterparts.[12,28,33,37] In fact, there is evidence that decellularized heart valve matrices will undergo partial autologous recellularization by native cells when implanted *in vivo*. Native heart valves are primarily populated by valve endothelial cells (VECs) on the luminal surface and valve interstitial cells (VICs) within the tissue matrix.[25] In both human trials and animal models, decellularized valves have demonstrated complete re-endothelialization by VECs and partial repopulation by VICs in the valve conduit wall and leaflet base; however, the distal leaflet remains acellular.[11,13,14,30] Therefore, it is theorized that *in vitro* repopulation of the interstitial distal leaflet combined with the aforementioned *in vivo* autologous recellularization will lead to complete cellular repopulation of a TEHV. Bone marrow mesenchymal stem cells (MSCs) are an appealing seeding cell source due to their phenotypic similarity to VICs and chemotactic potential to recruit autologous cells.[19] Once implanted, complete recellularization will likely be realized in a small part through the proliferation and differentiation of the MSCs and more significantly by the chemotactic recruitment of autologous cells.[3,4,7] However, decellularized scaffolds have shown difficulty with reproducibly establishing a phenotypically appropriate cell population within the leaflet matrix *in vitro*.[38] The importance of establishing a cell population *within* the distal leaflet tissue should be noted, as this is the region that does not demonstrate autologous recellularization and seeded cells remaining on the valve surface will be sheared away by blood flow post-implant. Thus, the cell seeding process should focus on establishing an interstitial cell population of MSC-myofibroblast lineage within the mid to distal valve leaflet matrix.

Enhancing the *in vitro* recellularization of the leaflet interstitial tissue may require fine-tuning of bioreactor conditioning culture parameters. Numerous studies using custom bioreactor systems have been used to apply mechanical and chemical stimuli to valve scaffolds to drive cell proliferation, differentiation, and ultimately recellularization.[5,10,23,24,32,34,36] Many of these systems employ physiologic parameters of pressure and flow rate to mimic a systemic or pulmonary circulation environment and have shown benefits including tissue maturation and re-endothelialization.[23,24,32,34,36] However, localization of cells within the distal leaflet of decellularized heart valve tissue remains challenging.[23,24,36] While it is inherently logical to use physiologic parameters for bioreactor conditioning, it may also be necessary to investigate novel, potentially non-physiologic (i.e. not similar to either the pulmonary or systemic circulation) conditioning parameters, in order to stimulate cell infiltration into the valve leaflet. One such solution is hypoxic conditioning of the seeded heart valves. Progenitor cells and mesenchymal stem cells cultured under low oxygen tension have demonstrated increases in cell proliferation and migration.[15,22,26] Additionally, polymeric valve scaffolds seeded with venous myofibroblasts cultured in hypoxic conditions exhibited increased mechanical properties compared to scaffolds cultured at normoxic conditions.[2] Another influence on recellularization may be cyclic chamber pressurization using positive and negative pressures. Previous work in our lab has demonstrated partial recellularization of the distal valve leaflet using cyclic positive and negative chamber pressure cycles within a novel bioreactor.[10] Ovine aortic valves seeded with human MSCs showed increased recellularization following a combination of negative and positive pressure conditioning, compared to negative conditioning only or static conditioning.[10] However, it was impossible to de-couple the effects of pressure conditioning from culture duration in the previous study. Therefore, the purpose of this study is to investigate the effects of non-physiologic conditioning parameters such as hypoxia and positive/negative cyclic pressures on the recellularization of heart valve leaflets, specifically cellular infiltration into the distal leaflet tissue.

MATERIALS AND METHODS

Tissue and Tissue Processing

Ovine aortic valves were harvested from juvenile sheep under approved IACUC protocols and in accordance with Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23). Harvested valves were cryopreserved using a procedure modeled after clinical tissue handling protocols. Valve were frozen at 1 °C min^{-1} using a controlled rate freezer (2100 Series, Custom BioGenics Systems) before being stored in a cryofreezer at − 180 °C. Valve decellularization was performed as described previously.[8] Briefly, the ovine aortic valves were thawed and then subjected to reciprocating osmotic shock, followed by two sequential detergents (Triton X-100, sodium-lauroyl sarcosine) and enzymatic (Benzonase) washes to remove cellular material. Extraction of organic material was performed using recirculating water and ion exchange

resins for up to 40 h. After decellularization, the valves were again cryopreserved using the above procedure and stored at − 180 °C until valve seeding.

Valve Seeding and Bioreactor Conditioning

Human cells were used for TEHV seeding since they provide translational results and allow characterization of the protein expression following bioreactor conditioning. All tissue engineered valves were seeded with human bone marrow derived MSCs. MSCs were isolated by filtering 25 mL bone marrow aliquots (Lonza) for the mononuclear cell fraction using a bone marrow filter system (Kaneka) and further isolated as the adherent cell fraction (passage 0). MSCs were then expanded in culture according to their destined TEHV conditioning group: MSCs for valves using hypoxic bioreactor conditioning were cultured in hypoxic conditions (7% O_2, 5% CO_2, 37 °C), while MSCs for valves using normoxic bioreactor conditioning were cultured in normoxic conditions (21% O_2, 5% CO_2, 37 °C). Two cell populations were isolated from a single donor with one of the matched populations being used for a hypoxic conditioning group and the other used for a normoxic conditioning group, effectively creating matched cell populations that differ based on oxygen tension conditioning. The cells were passaged once during culture to obtain sufficient numbers for valve seeding. Hypoxic cultured cells often reached confluency more quickly than normoxic conditioned cells, so cell culture duration varied between groups, however all cells were harvested for seeding at passage 2.

The valves were seeded following our previously established protocols.[10] The decellularized valves were thawed from cryopreservation and sutured onto tissue grips. 10 million cells from the appropriate MSC population were then suspended in 10 mL of media and seeded into the lumen of the valve, which was mounted in a static bioreactor chamber containing 200 mL valve media [DMEM F12 (Life Technologies) and 10% human serum (Sigma-Aldrich)]. Three valves were used for each of the four groups to evaluate bioreactor conditioning parameters: hypoxic and high pressure (Hyp/HighP), normoxic and high pressure (Norm/HighP), hypoxic and negative pressure (Hyp/NegP), and normoxic and negative pressure (Norm/NegP). After cell seeding, all valves underwent 24 h of static culture (37 °C, 5% CO_2) to allow cell adhesion. Valves were then transferred to the sterile, single use, pulsatile bioreactor chamber containing 500 mL valve media and the assembly was placed on a linear actuator to create cyclic positive and negative pressure profiles within the bioreactor chamber.[10] The appropriate bioreactor conditioning protocols were then applied for 2 weeks based on the respective groups. Valves in the hypoxic groups were cultured at 7% O_2 and valves in the normoxic groups were cultured at 21% O_2 (all valves cultured at 37 °C, 5% CO_2). Valves in the high pressure groups were conditioned for 1 day of cyclic negative pressure (− 20 to 5 mmHg), followed by 1 day of cyclic low positive pressure (− 5 to 20 mmHg), and finally 14 days of cyclic high positive pressure (− 5 to 120 mmHg). Valves in the negative pressure groups were conditioned for 16 days of cyclic negative pressure (− 20 to 5 mmHg). Examples of the cyclic pressure profiles and pressure experienced within the lumen of the valve have been published previously.[10] A media change was performed on all valves after 1 week of conditioning, wherein half of the bioreactor media was removed and replaced with fresh valve media. Following bioreactor conditioning the valves were removed and dissected for analysis, including histology, cell counting, immunohistochemistry (IHC), and biochemical analysis.

Histology and Cell Counting

Samples for histology were sectioned along the radial plane of each leaflet from each of the tissue engineered valves. Hematoxylin and eosin (H&E) staining was used to evaluate repopulation of the decellularized valve leaflets, specifically by the presence of cells within the leaflet tissue. Recellularization was measured as the density of infiltrated cells within the interstitial tissue of each leaflet (n = 9 per group). Cells were counted within a calculated area using Axiovision software and cell density was calculated at the base, middle, and distal free edge region of the leaflet. Figure 1a provides an example of the regional breakdown of the valve leaflet. Cells on the leaflet surface were not included.

Immunohistochemistry

Protein expression of the cells from the tissue engineered valves was evaluated by IHC. Unstained slides were cut from the samples prepared for histology. The slides were blocked in 10% normal goat serum for 1 hour before overnight incubation at 4 °C with 1:100 diluted primary antibodies. Primary antibodies from mice or rabbits (Abcam) targeted alpha smooth muscle actin (αSMA), heat shock protein 47 (HSP47), vimentin (VIM), CD90, CD73, and von Willebrand Factor (vWF). Goat anti-rabbit (Alexa Fluor 594, Life Technologies) and goat anti-mouse (Alexa Fluor 488, Life Technologies) secondary antibodies were then incubated for 1 h followed by nuclear counterstaining (DAPI; Life Technologies). Slides were imaged within 24h of staining.

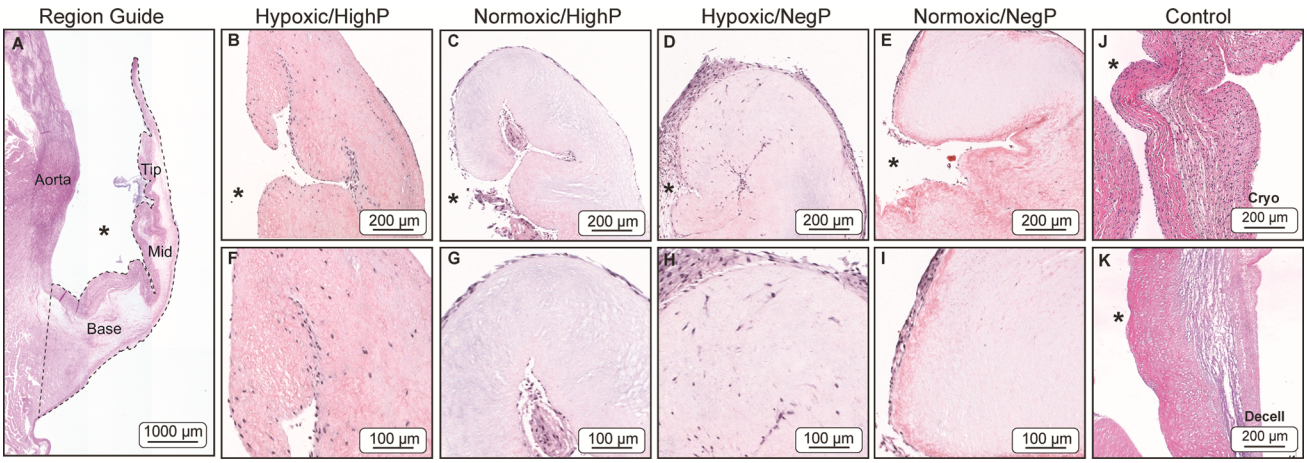

FIGURE 1. Representative H&E stained sections of valve leaflets. The Region Guide (a) provides an overview and demonstrates the zones for the tip, mid, and base regions of the valve leaflet with dashed lines indicating the boundaries for counting infiltrated cells. Note that only infiltrated cells were counted and cells on the valve surface were not included in counting. Images taken from the tip region of Hypoxic/HighP (b, f), Normoxic/HighP (c, g), Hypoxic/NegP (d, h) and Normoxic/NegP (e, i) groups indicate varying degrees of cellular infiltration. Note that images (f–i) are magnified images of (b–e), respectively, to visualize cellular infiltration. Positive and negative controls (j and k, respectively) are presented as cryopreserved (cryo) and decellularized (decell) ovine valve samples. Both the Hypoxic/HighP valves and the Hypoxic/NegP valves exhibited a high degree of recellularization with cells infiltrated into the leaflet matrix. Conversely, Normoxic/HighP valves and Normoxic/NegP valves exhibited minimal cellular infiltration. * indicates the outflow side of the leaflet.

Biochemical Analysis

The sulfated glycosaminoglycan (GAG), collagen, and total protein concentrations of the extracellular matrix from each of the tissue engineered valve leaflets ($n = 9$ per group) was analyzed using colorimetric biochemical assay kits. GAG concentrations were measured using the Blyscan Sulfated Glycosaminoglycan Assay (Biocolor). Tissue samples (approx. 15 mg) were processed according to manufacturer protocol and the results were measured using a UV/Vis spectrophotometer (VERSAmax, Molecular Devices). Collagen concentration was measured using the QuickZyme Total Collagen Assay (QuickZyme Biosciences). Samples (approx. 15 mg) were prepared by overnight hydrolysis in 6M HCl at 90 °C and the hydroxyproline concentration was measured using a spectrophotometer. Total protein concentration was measured using the QuickZyme Total Protein Assay (QuickZyme, Biosciences) and used the same hydrolysates that were prepared for collagen quantification. The GAG, collagen, and total protein concentrations are reported as $\mu g\ mg^{-1}$ of wet tissue and are compared to previously reported values from cryopreserved and decellularized valves.[39]

Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA), and post-hoc comparisons were made using the Tukey test for parametric data. The Kruskal-Wallis post-hoc comparison was used for non-parametric data, specifically the cell counting measurements. Further statistical comparisons between hypoxic/normoxic groups within a given pressure conditioning, particularly for the cell density measurements, were made using the Mann–Whitney Rank Sum test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Tissue, and Tissue Processing

As reported previously, the decellularization process resulted in complete removal of cells from the valve tissue while preserving the overall architecture of the extracellular matrix (Figs. 1e and 1j).[9,39] Following the two week bioreactor conditioning protocols the valves from all groups had a normal appearance and exhibited complete coaptation between the leaflets.

Histology and Cell Counting

H&E staining of the tissue engineered valve samples revealed the four bioreactor conditioning protocols led to differing degrees of recellularization (Fig. 1). Valves in the Hypoxic/HighP group and Hypoxic/NegP group showed increased cellularity within the leaflet interstitium (Figs. 1a, 1f, 1c, and 1h). Particularly the distal region of the leaflet, which exhibited the greatest cell concentration, while the middle region and base of the leaflet showed less cellular infiltration. Valves in the

Normoxic/HighP and Normoxic/NegP groups exhibited less cellularity within the interstitial leaflet tissue, but showed a high degree of cells clumped together along the leaflet surface (Figs. 1b, 1g, 1d, and 1i). Similar to the hypoxic groups, the highest cell concentration was on the distal leaflet with decreased cellularity along the leaflet middle and base. The variable pressure conditions led to no obvious differences in recellularization between groups.

The amount of recellularization within each group was quantified by measuring the cell density within the leaflet tissue (Fig. 2). All groups had an increased cellularity in the distal leaflet compared to the middle or base regions. Since the goal of this study was recellularization of the distal leaflet the analysis will focus on the cell concentration within the distal third or "tip" region. Hypoxic conditioning led to increased cell density compared to normoxic conditioned valves. The Hypoxic/NegP group exhibited relatively high levels of recellularization with a median cell density of 116.34 cells/mm^2 within the leaflet tip. The Hypoxic/HighP group also exhibited high recellularization with one sample having the greatest recellularization, however the results were not consistent leading to high variance and a median cell density of 58.66 cells/mm^2. The Norm/HighP and Norm/NegP had relatively low recellularization with median values of 20.64 and 26.01 cells/mm^2, respectively. Statistical analysis by ANOVA on ranks indicated a significant difference was to be expected ($p = 0.045$), but the post-hoc comparison found no significance between individual groups. Further statistical analysis was performed using the Mann–Whitney test between hypoxic and normoxic samples within the individual pressure conditioning groups (i.e. negative pressure or high pressure). Using this analysis method, hypoxia led to significantly increased cell density compared normoxic conditioning for both negative pressure ($p = 0.030$) and high pressure ($p = 0.042$) groups.

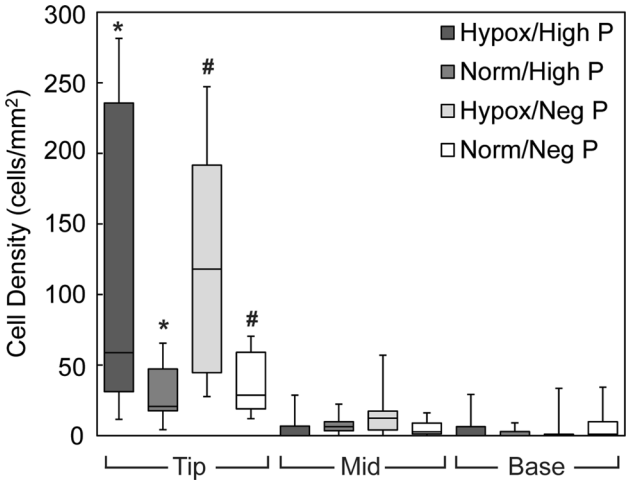

FIGURE 2. Box and whisker plot of the cellular density of the tissue engineered valve leaflets from the various bioreactor conditioning groups ($n = 9$ per group). Cellularity was measured at the tip, middle, and base regions of each leaflet. The box boundaries represent the first and third quartiles, the horizontal line indicates the median, and the whiskers indicate the maximum and minimum values. Significance was analyzed between hypoxic/normoxic conditioning, within individual pressure conditioning groups. Symbols of the same type (*, #) indicate significance between groups ($p < 0.05$).

Cell Phenotype Analysis

The phenotype of the cells within the tissue engineered valves was evaluated by protein expression using IHC (Fig. 3). Native valve interstitial cells that are actively remodeling matrix display a myofibroblast-like phenotype. The protein expression of our seeded cells was very similar between groups, with all groups displaying markers indicative of MSC or VIC phenotypes. Specifically, cells in all groups displayed positive expression of the MSC marker CD90 as well as the VIC-like markers αSMA, HSP47, and VIM. The Hypoxic/HighP group also exhibited positive expression of CD73, another MSC marker. None of the groups displayed positive expression of the endothelial cell marker vWF.

Biochemical Analysis

The concentration of GAG, collagen, and total protein in the extracellular matrix of the tissue engineered valves was measured and compared to previously reported values for cryopreserved and decellularized samples (Table 1).[39] The biochemical analysis revealed no differences between the bioreactor conditioning groups. As reported previously, the decellularization protocol causes a significant decrease in GAG concentration. However, none of the tissue engineered valves had a significantly different GAG concentration than either decellularized or cryopreserved controls, indicating some amount of GAG production by the seeded cells. On the other hand, the collagen concentration of all tissue engineered valves from each group was significantly greater than both the cryopreserved and decellularized controls ($p < 0.05$). The concentrations of total protein in the tissue engineered valves revealed no significant differences between groups or between the cryopreserved or decellularized controls. The measured collagen concentration between the decellularized and tissue engineered samples indicates a level of collagen production that is much greater than has been observed by others.[31] This effect is not an artifact of tissue mass since the total protein concentrations did not demonstrate a similar trend. Others have demonstrated that under cyclic strain bone marrow MSCs can double the amount of collagen production compared to static

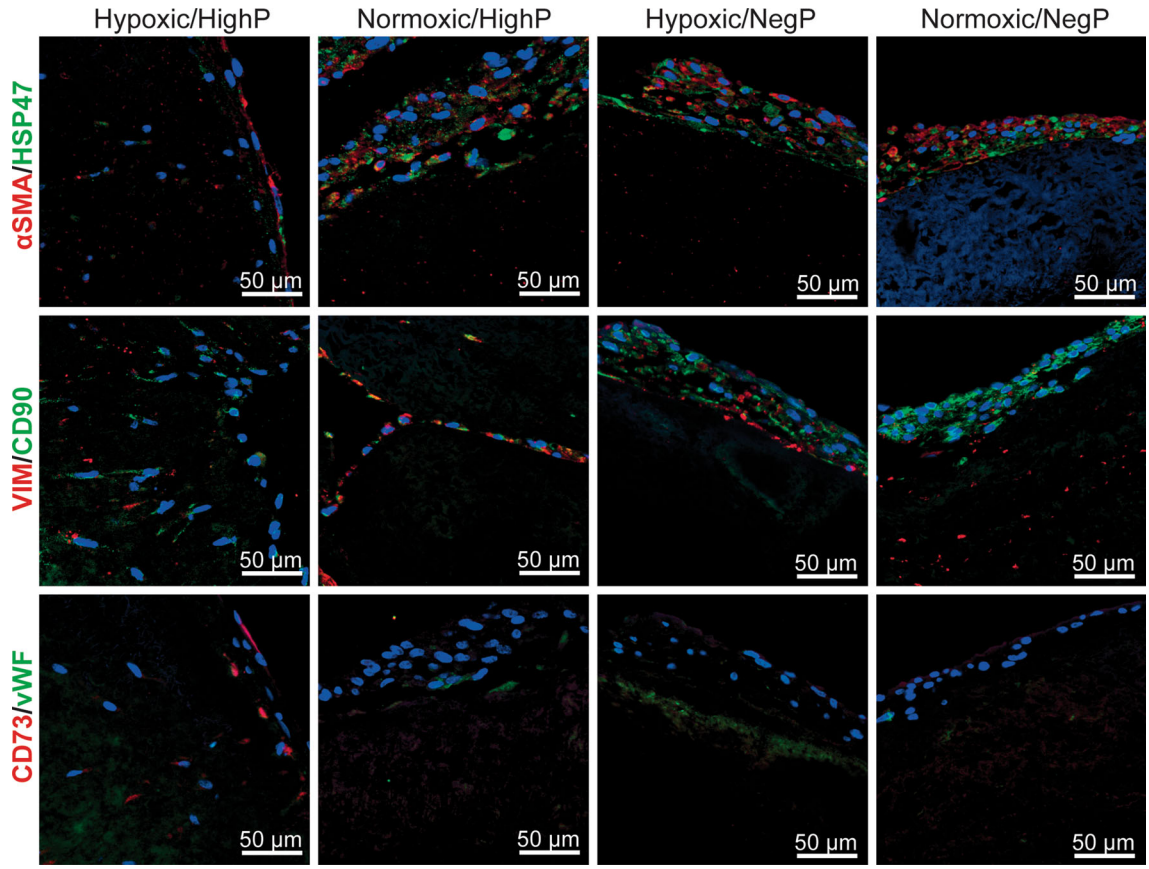

FIGURE 3. Immunohistochemical sections of bioreactor conditioned valve leaflets dual stained for the expression of: cell nuclei (blue) (all), αSMA (red) & HSP47 (green) (a–d), VIM (red) & CD90 (green) (e–h), and CD73 (red) & vWF (green) (i–l). All groups show positive expression for αSMA, HSP47, VIM, and CD90. The Hypoxic/HighP group shows positive expression for CD73. None of the groups stained positive for vWF.

TABLE 1. Biochemical Content of tissue engineered heart valves.

	GAG (μg mg^{-1} wet tissue)	Collagen (μg mg^{-1} wet tissue)	Total protein (μg mg^{-1} wet tissue)
Hypox/HighP	0.649 ± 0.173	54.59 ± 15.64^{a,b}	131.93 ± 44.44
Norm/HighP	0.427 ± 0.204	63.61 ± 13.54^{c,d}	139.47 ± 38.28
Hypox/NegP	0.516 ± 0.172	50.17 ± 9.32^{e,f}	106.20 ± 35.13
Norm/NegP	0.474 ± 0.157	53.43 ± 4.90^{g,h}	128.47 ± 32.88
Cryo	1.622 ± 1.199^{a}	25.78 ± 2.85^{a,c,e,g}	153.47 ± 46.65
Decell	0.390 ± 0.473^{a}	24.52 ± 7.72^{b,d,f,h}	133.42 ± 64.27

Concentration of GAG, collagen, and total protein measured within the extracellular matrix of the tissue engineered heart valve groups. Data is presented as the mean ± standard deviation ($n = 9$). Previously reported data for cryopreserved (cryo) and decellularized (decell) ovine aortic valves are included for comparison.[39] Superscript letters of the same type indicate a significant difference between groups ($p < 0.05$).

conditions, though this likely does not account for the unexpected amount of collagen production observed herein and suggests further investigation.[7,31]

DISCUSSION

The use of *in vitro* bioreactor conditioning has been well explored for heart valve tissue engineering, and conditions mimicking the physiologic environment have been shown to positively effect cell differentiation and tissue maturation.[32] However, culture at physiologic parameters directly after seeding has also been shown to be detrimental, such as a high flow rate which can shear away attached surface cells.[23] The use of non-physiologic parameters, either ramping up to physiologic conditions or novel parameters, can be beneficial to cell infiltration and recellularization. This study investigated the interactive effects of two non-

physiologic parameters on the *in vitro* recellularization of tissue engineered heart valves seeded with MSCs, oxygen tension and cyclic pressure conditioning. Histologic evaluation and cellular density measurements determined that conditioning valves under hypoxia increased cell infiltration in the valve leaflet, particularly within the tip (distal third) of the leaflet. Protein expression revealed a relatively similar cell phenotype of MSC or VIC-like cells within all groups, indicating that the bioreactor conditioning did not adversely affect the cell phenotype. Biochemical concentrations of extracellular matrix proteins were also similar between all groups. The results demonstrate that the tested bioreactor conditioning parameters, particularly hypoxic culture, can positively influence MSC infiltration into decellularized heart valves.

As mentioned, the objective of increasing MSC infiltration into the distal leaflet *in vitro* may act twofold in order to achieve a fully recellularized valve once implanted *in vivo*: seeded MSCs may proliferate and differentiate into the appropriate VIC phenotype or the seeded MSCs may increase autologous cell recruitment into the distal leaflet through chemotaxis. It is necessary that cells infiltrate the leaflet basement membrane since this is the region that does not see innate autologous recellularization and to prevent cell loss due to blood shear once implanted. Other groups have investigated the outcome of MSC seeded heart valves *in vivo* using animal models. Vincentelli *et al.* injected MSCs into the conduit of decellularized pulmonary valves implanted in the right ventricular outflow tract of sheep.[40] They found injected MSCs were still present at one week along with host cells and the seeded valves functioned well out to four months.[40] However, a similar study by Harpa *et al.* observed conflicting results in which the valves with injected MSCs developed a fibrotic inflammatory response while non-cell seeded valves did not.[16] Despite these two conflicting studies, other groups have demonstrated success with bone marrow derived cells seeded on implanted valves, suggesting there is a benefit to cell seeding.[20,21]

Effects of Cyclic Pressure Conditioning

Our group has previously demonstrated that a similar bioreactor system can be used to condition heart valves in a low flow, cyclic pressure protocol leading to increased recellularization compared to static culture.[10] This bioreactor system uniquely utilizes cyclic negative and/or positive chamber pressures within the bioreactor, which was discovered to promote cellular infiltration during initial protocol development. In this present study, we found the use of negative cyclic pressures vs. a combination of negative and positive cyclic pressures lead to similar degrees of the recellularization indicating the negative pressure may be required for the recellularization response. While the exact mechanism remains unclear, it is theorized that the negative pressure cycles increase saturation of the leaflet with cell culture media and/or causes increased cell deformation, mimicking diastolic loading.[27] These putative mechanisms are not unlike the proposed mechanisms that accelerate tissue healing with cyclic negative pressure wound treatment.[18]

Effects of Oxygen Tension Conditioning

Early work into the effects of hypoxia on MSCs was focused on tumor angiogenesis and found that cell migration was greatly increased under hypoxic conditions.[1] More recent work has focused on using hypoxia to increase the therapeutic effects of MSCs for tissue engineering.[6,41] Such studies have found hypoxia increases the production of hypoxia-inducible factor 1α (HIF-1α) in MSCs, which further mediates stromal cell derived factor 1 (SDF-1) production and ultimately cell migration.[41] This is in agreement with the results of this study which demonstrate that hypoxic conditioning can increase valve recellularization, likely through increased cell migration. Since one hypoxic valve and one normoxic valve were generated from the cells of each donor, we can compare donor-matched hypoxic and normoxic valves (Table 2). Hypoxic conditioning led to increased leaflet recellularization for each matched pair of valves from a single donor, regardless of pressure conditioning. Additionally, donor cell variation can be observed as valves showing the greatest cell infiltration within each group were donor matched (e.g. Donor 1 and Donor 5). The positive pressure conditioned valves exhibited a large variance in the fold change with one pair of valves exhibiting greater than 8-fold increase with hypoxic conditioning while the other two pairs of valves exhibited only a moderate increase. Conversely, negative pressure conditioned valves experienced at least a 2-fold increase in cell density due to hypoxia in all valves. These results may suggest synergistic effects on leaflet recellularization between hypoxia and negative pressure conditioning.

Hypoxic conditioning significantly promoted cellular infiltration of MSCs into the valve leaflet, yet the large variability within the hypoxic conditioned groups also indicates the inconsistent nature of TEHV recellularization. As we have reviewed previously, reproducible establishment of a phenotypically appropriate cell population within the leaflet tissue remains challenging.[38] Such inconsistency led to samples within this study demonstrating a level of recellularization similar to native leaflets, while other identically treated samples exhibited only marginal cellular repopulation.

TABLE 2. Interstitial cell density of the tissue engineered heart valves.

Donor	Pressure conditioning	Hypoxic valve cell density (cells mm^{-2})	Normoxic valve cell density (cells mm^{-2})	Fold change
Donor 1	Positive	259.53 ± 22.96	30.96 ± 30.09	8.38
Donor 2	Positive	44.92 ± 13.75	38.41 ± 17.63	1.17
Donor 3	Positive	41.11 ± 19.00	22.80 ± 19.82	1.80
Donor 4	Negative	38.25 ± 9.41	18.18 ± 13.83	2.10
Donor 5	Negative	160.93 ± 106.26	70.38 ± 0.06	2.29
Donor 6	Negative	142.35 ± 42.27	24.70 ± 19.81	5.76

Measured cell densities at the distal tip region of each donor matched hypoxic and normoxic valve. Measurements were made on a per leaflet basis. Data is presented as the average per valve ± standard deviation. The fold change displays the cell density of the hypoxic valve compared to the normoxic valve.

Despite the large variance, hypoxic conditioning still demonstrated a significant increase in recellularization of heart valve leaflets seeded by MSCs compared to normoxic conditioning. Notably, recellularization occurred mostly within the distal tip region of the valve leaflets. All other regions of valvular tissue (leaflet middle, leaflet base, and conduit wall) have shown evidence of autologous recellularization during the implantation of decellularized valves in human and animal models.[11,13,14,30] Since the distal leaflet region has previously proven difficult to repopulate, the use of hypoxia to increase *in vitro* MSC recellularization appears to be an important variable to further explore relative to TEHVs.

Limitations

One of the primary limitations of this study was the small sample size within each group. Twelve aortic valves were processed, three within each group, providing nine leaflets within each group available for analysis. While nine samples was adequate to detect the significant effects of hypoxic conditioning, the variance within the measured population was still very high. There is also concern when using leaflets from a single valve as discrete samples since valve age and biologic variability may affect recellularization. For completeness, a similar statistical analysis was performed after pooling the leaflets and the samples were analyzed on a per valve basis ($n = 3$ per group for cell counting and biochemical analysis), rather than a per leaflet basis. In such an analysis, the variation within groups is much greater, so a logarithmic transformation was applied, followed by ANOVA between groups. Analysis in this manner resulted in significant differences between the Norm/NegP and the Hypox/HighP ($p = 0.02$) and Hypox/NegP ($p = 0.04$) groups. However, we believe the analysis of each individual leaflet within this study is valid. The valve to valve biologic variability was reduced within this study by obtaining all valves from the same strain of similarly aged, juvenile sheep and followed an identical decellularization protocol. In addition, following *in vivo* chronic implantation we have observed independent wear, remodeling, and variability in recellularization from leaflet to leaflet within the same implanted valve.[29,30] Therefore, in this context it is reasonable to consider each leaflet a discrete sample for the analysis described in the methods.

Another limitation of this study is the use of decellularized xenogeneic heart valves seeded with human cells. Based on the challenges seen in decellularizing animal tissue, particularly heart valves, using decellularized allogeneic heart valves would be preferred but remains challenging due to the limited human tissue available for research.[37] However, it is unlikely that using the ovine tissue caused any unforeseen benefits within this study, and similar or better results might be expected using decellularized allografts. Finally, this study is limited by the use of a single time point for measurements rather than serial sampling. All tissue engineered valves were processed for 16 days based on previous experience with cell seeded valve constructs. However, that time point is largely arbitrary and further work is required to determine the ideal duration of bioreactor processing. Regardless of the limitations, the results of this study provide intriguing leads for increasing the cellular infiltration of seeded decellularized valve scaffolds. Future studies should focus on fine-tuning the bioreactor conditioning protocol based on hypoxia and a negative pressure waveform.

CONCLUSION

Adjusting the bioreactor conditioning parameters for cyclic negative/positive chamber pressure and oxygen tension resulted in varying degrees of recellularization for MSC seeded TEHVs. Conditioning in a low oxygen environment led to increased cellular infiltration into the valve leaflet tissue compared to normoxic culture. The use of cyclic chamber pressur-

ization has been shown previously to increase cellular infiltration, but the pressure conditioning protocols tested herein did not lead to differences in recellularization. These results provide evidence that bioreactor conditioning of tissue engineered constructs need not always mimic physiologic conditions, and that it is worth investigating novel or uncharacteristic culture conditions to benefit aspects of tissue culture, in this case cellular infiltration. Further development of such protocols may increase the reproducibility in the recellularization of decellularized valve constructs and ultimately the translation of a TEHV.

CONFLICT OF INTEREST

M. VeDepo, E. Buse, A. Paul, G. Converse, and R. Hopkins declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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