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EFFECTS OF OSCILLATORY FLOW PATTERNS ON RAT VALVULAR ENDOTHELIAL AND INTERSTITIAL CELLS

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To: Dr. Steven Oberbauer, Chairperson Department of

Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Alexandra Tchir entitled " Effects of Oscillatory Flow Patterns on Rat Valvular Endothelial and Interstitial Cells", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor have read this thesis. We recommend that it be approved.

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ABSTRACT

Valve endothelial cells (VECs) and the underlying layer of valve interstitial cells (VICs) are critical regulators of the heart valve homeostasis and pathologies such as valve calcification. Valvular endothelial cells sense cues from their environment and send signals to interstitial cells to regulate their response. One critical mechanical regulator is blood, which can have a unidirectional or oscillatory flow. These different patterns can have various effects on cells. This study seeks to determine if oscillatory flow characteristics applied to VECs will change the communication factors to valve interstitial cells. A series of oscillatory flow profiles were applied to the VECs. The spent media was collected containing signaling molecules. Valvular interstitial cells were grown in the oscillatory flow conditioned media from VECs to determine the effect of the signals. The calcium deposits left by the interstitial cells were stained and quantified. The most calcification was seen in interstitial cells that received equal amounts of pro-calcific media and the conditioned media from endothelial cells exposed to the highest amount of oscillatory flow. The calcification was substantially lowered in interstitial cells that were grown in media from endothelial cells conditioned in steady flow and intermediate oscillatory flow. We propose that onset of valve calcification may be paracrine regulated (VECs-to-VICs) at high oscillatory flow locations (OSI = 0.50) when regional calcium concentration levels are already augmented. Hence, further discoveries in this paracrine pathway will be important in identifying potential therapeutic targets for valve calcification.

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INTRODUCTION

Aortic heart valve disease affects about 10.7% of the population in America aged 65 and older (Bach et al. 2007). Aortic sclerosis, the most prevalent form of heart valve disease is a stiffening of the heart that progressively leads to stenosis, a condition that affects the ability of the valve to open fully and reduces blood flow to the body (Faggiano et al. 2006). Calcific aortic stenosis is the most common form of stenosis in developed countries and is characterized by the buildup of calcific nodules and structural deformities on the heart valve leaflet (Lindman et al. 2016). High levels of serum calcium (2.63 ± 0.28 mmol/L), phosphate (1.56 ± 0.33 mmol/L) and calcium-phosphorous products (4.16 ± 1.13 mmol/L) are associated with calcific aortic stenosis (Akat et al. 2010). Heart valve replacement surgery for aortic stenosis is the second most common cardiac surgery performed (Roberts & Ko 2005). Mechanical heart valves are a sturdy replacement option and can last beyond 25 years, however a patient would need to take daily blood thinners (Lindman et al. 2016). Bioprosthetic heart valves are derived from porcine or bovine sources and have near human hemodynamic conditions. However, they have about a 10-year shorter lifespan than mechanical valves and are at risk of calcification and stenotic failure (Leopold 2012). While valve replacements are traditionally implanted using open heart surgery, trans aortic valve replacement (TAVR) is a less invasive, catheter-based approach. This technique uses a inserts a bioprosthetic valve, however clotting, regurgitation and other complications have been reported (Lindman et al. 2016). A better understanding at the cellular level and the mechanisms behind homeostasis and disease might lead to better solutions for valvular replacements. The valvular leaflets are composed of an outer layer of endothelial cells and populated within by interstitial cells which are responsible for structural integrity and composition (Rutkovskiy 2017). Endothelial cells have a multitude of functions including

maintaining hemostatic balance, vascular tone, angiogenesis, wound healing, smooth muscle proliferation and inflammation (Widmer & Lerman 2014). The endothelial cells receive signals from both intrinsic and extrinsic factors and responds in different ways to maintain homeostasis (Widmer & Lerman 2014). A major regulator of the valvular endothelium are hemodynamic mechanical forces. Cyclic strain from changes in the vessel diameter, circumferential pulsatile pressure, blood pressure, and shear stress have all been found to affect the regulation of endothelial cells (Isales 1993). Shear stress, the tangential force of blood flow on the surface of the blood vessel, is considered the most crucial hemodynamic factor that affects endothelial cells (Davies 2008). Endothelial cells have mechanoreceptors which sense the flow of blood circulation, transduction of these signals can lead to deeper structural changes in the interstitial cells (Chistiakov et al. 2017).

Pulsatile blood flow generates oscillatory patterns at different sites in the cardiovascular system, resulting in oscillatory shear stresses. A parameter used to quantify oscillatory flow is the oscillatory shear index (OSI). The OSI is used to quantify the magnitude and directionality of the fluid-induced shear stress and the development of the oscillations over time. The oscillations result from the main vector component of fluid flow changing from its principal direction (Ku et al. 1985). Oscillations and low shear stress can occur at bifurcations and sharp turns in the vascular system (Ku et al. 1985). Changes in flow can occur on a very localized scale, with phenotypic changes confined to affected regions (Chistiakov et al. 2017). For example, the flow patterns on either side of the valvular leaflet differ. The endothelial cells on the aortic side of the valve constantly experience flow reversal resulting in oscillations (Cao et al. 2016). These cells are more prone to calcification than the other (Simmons et al. 2005).

Current research, including *in vitro* and clinical studies, states that protective and diseased phenotypic states have distinct flow types. High shear stress is said to enhance mass transport of material, such as cholesterol, away from the vascular wall, although there are other factors involved, such as resistance of the material to diffusion (Caro et al. 1971). In addition, the downregulation of atherogenic genes is seen in straight regions of the vascular system, where there is unidirectional flow with high shear stress (Chiu & Chien 2011). While the range can differ depending on the location in the cardiovascular system, high shear stress is characterized on the order of 100–8000 dyne/cm^2 (Akins 1995; Kroll 1996; Wootton & Ku 1999). Within the valvular environment, simulations have shown shear stress on the aortic side ranges between and 0 to 21.3 dyne/cm and 0 to 71 dyne/cm on the ventricular side. (Yap et al. 2012a). A contrasting study estimates a range of -8 to 10 dyne/cm on the aortic surface (Sun et al. 2011). In the vascular system, highly localized disturbed flow is generally associated with pathological conditions and results in average low shear stress with high oscillatory flow (Davies 2008). Early sites of atherosclerosis often appear in non-random patterns, in known disease-prone regions where the architecture leads to disturbed blood flow (Chiu & Chien 2011). For example, pathogenesis is observed in carotid bifurcations where fluid velocity simulations of the same sites show combined low shear stress and high oscillations overlap with sites of high intimal thickness (Ku, et al., 1985).

Recently discovered marked differences in structure and function between vascular and valvular endothelial cells, such as cell type, cellular organization, and response to strain, are now leading researchers to believe that the endothelial cells on vessels and valves have different specializations and characteristics (Deck et al. 1988; Butcher et al. 2004; Ferdous et al. 2011). Butcher et al. (2004) found that when exposed to a steady flow, vascular cells align in parallel

while valvular cells align perpendicularly. In addition, alignment in vascular and valvular endothelial cells was dependent on different signaling pathways. The differences between the functionality of the cell types may be a result of conditioning from the different environmental stresses the two systems are exposed to. Engelmayr et al. (2006) looked at the effect of two mechanical factors of the heart valve environment, cyclic flexure and unidirectional flow. Cyclic flexure, the pattern of the opening and closing of the valves, produces oscillations and laminar flow, the fluid shear stress from blood flow. Human bone marrow-derived mesenchymal stem cells (hBMSCs) were tested both in coupled and independent conditions. Unidirectional flow alone in the system did very little for the development of the proper cellular phenotype. After 3 weeks exposed to both flex and flow there were signs of endothelial cell markers and interstitial cell markers. A collagen assay showed significantly increased collagen and results from histology and scanning electron microscopy showed substantially increased tissue formation with the coupled stimuli, flex-flow. The existence and influence of oscillatory flow as a contributing biomechanical parameter was demonstrated in stem cells that were being conditioned to differentiate to valvular cells (Salinas and Ramaswamy 2014). Physiological pulsatile flow conditions were compared with non-physiological pulsatile flow and flex-flow conditions to determine a range of OSI favorable to the valvular phenotype. Growing human bone marrow stem cells in the three ranges of OSI showed the best genotypical and phenotypical expression was in the range of physiological pulsatile flow conditions, which was 0.23 OSI. This suggests that there is an optimal range of oscillations and shear stress for a valvular phenotype (Williams et al. 2017).

Research on flow patterns is lacking in evaluating the effect of oscillatory flow isolated from low shear stress. Maintaining the shear stress magnitude while systematically modifying

localized OSI conditions, may help delineate distinct ranges of oscillatory blood fluid-induced biomechanical stimuli that may play a critical role in normal versus abnormal valvular tissue remodeling. This study sought to determine if there was a specific range of oscillatory shear stress that leads endothelial cells to signal calcification in interstitial cells separate from a range necessary to maintain valvular homeostasis. To accomplish this, a series of oscillatory flow profiles were applied to valvular endothelial cells (VEC). The media containing the biomarkers from the VECs conditioned in various flow types was collected and used to grow valvular interstitial cells (VIC). This experiment was done with media mimicking both a healthy environment and a pro-calcific environment. This determined the effects of oscillatory flow on both cells and its association with more healthy or diseased phenotypes.

MATERIALS AND METHODS

Conditioning of valve endothelial cells

Rat valvular endothelial cells were obtained from Celprogen Inc. (Torrance, CA) and maintained using the rat valvular endothelial primary cell culture media with serum grown in T75 flasks that had a complete extra-cellular matrix coating (Celprogen Inc.). Media was changed every two days and the cells were split and passaged every four days. Cell passaging is a way of splitting a single cell plate into two plates to allow exponential growth (Masters & Stacey 2007). The cells were plated to a size of 2×10^5/channel for a total of 8 channels/experimental group in a microfluidic shear stress cell assay system (Bioflux 200, Fluxion Biosciences, San Francisco, CA). To seed the Bioflux plates, cells were pumped through the microfluidic channels in the device. Then, fresh media was added, and the plates were placed in an incubator at 37 °C where the cells continued to grow under these conditions for 24 hours for cell attachment. To check that the cells were properly populated, the channels were examined using the viewing chamber on the microfluidic plate using an inverted microscope. Fresh media was added to the inlet wells of the channels and the device was configured to execute one of the flow profiles, oscillatory shear stress was represented using OSI (Eq. 1; Eq. 2). Three patterns of flow were applied through pneumatic pressure to the fluid media covering the cells, generating patterns of oscillations (Fig. 1). The profiles were applied for a period of 48 hours as follows: 0 (steady flow) OSI, 0.25 OSI, 0.50 OSI. An accompanying no flow control (static) group consisting of roughly 80,000 cells per channel was also cultured for the 48-hour period. Each group had a sample size of three.

$$OSI = \frac{1}{2}\left(1 - \frac{abs\left(\int_0^T \tau_W dt\right)}{\int_0^T abs(\tau_W) dt}\right)$$ Eq.1

*Oscillatory shear index (OSI). The duration of the cycle is T and wall shear stress is τ_W (Ku et al. 1985).

$$\tau_W = \mu \frac{\Delta V}{\Delta r}$$ Eq. 2

*Wall shear stress, τ_W. Absolute fluid viscosity is μ, the velocity parallel to the wall is V, and the radial distance to the vessel wall is r (Ku et al. 1985).

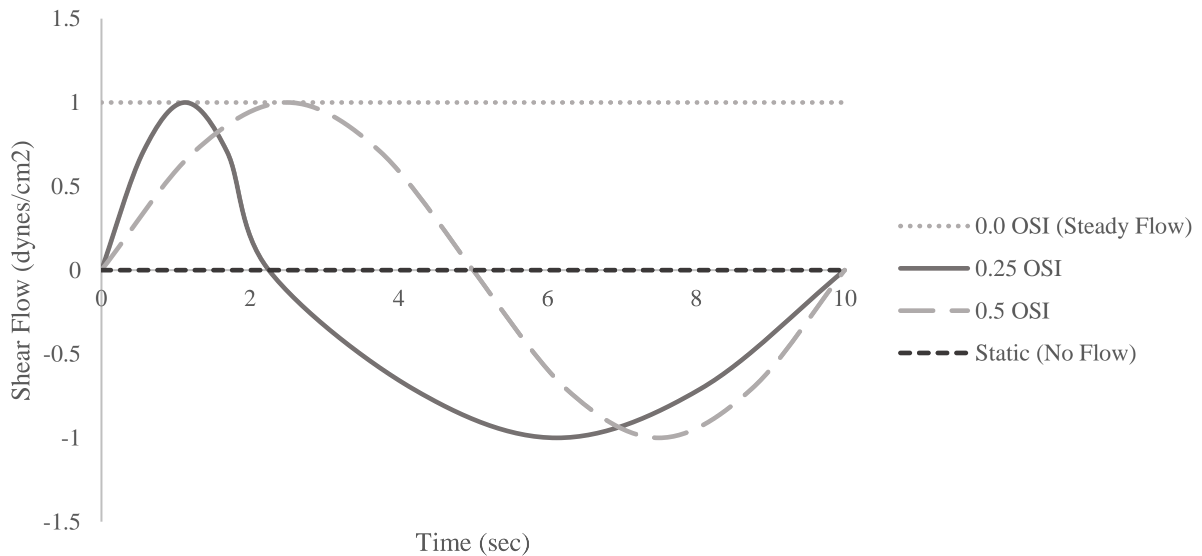

Figure 1. The four oscillatory shear index waveforms used to condition the VECs. An OSI of zero indicates a unidirectional forward flow, whereas 0.50 OSI represents a completely even oscillation.

Note that the microfluidic channels were pre-coated with gelatin for proper cell adhesion and subsequently primed with fresh media. The time-averaged shear stress in the flow experiments was in the order of 1 dyne/cm^2, with the steady shear stress group set to a constant value of 1 dyne/cm^2. A new microfluidic plate with cells was used for each waveform profile using three

replicates each. Next, a gene expression analysis was performed to evaluate several genes involved in valve endothelial cell function. After 48 hours of conditioning, RNA was extracted from the cells using TRIzol (Invitrogen, Carlasbad, CA), and *Power* SYBR Green RNA to CT *1-step* kit (Applied Biosystems, Waltham, MA) was utilized to perform RT-PCR (Applied Biosystems, Waltham, MA). The housekeeping gene used was *CDKN1B* and target genes were*, NOTCH1, VWF* and *ELN*. Using the CT values from the RT-PCR, the normalized gene expression for each flow profile was performed using the ΔΔCT/Livak method (Schmittgen & Livak 2008). After conditioning, the used media containing paracrine signals was collected from each group and frozen for later use.

Treatment of valve interstitial cells

Rat valvular interstitial cells (VICs) were obtained from Innoprot (Innoprot, Derio, Spain). The rat VICs were maintained using regular cell culture media consisting of Dulbecco's Modified Eagle Medium (DMEM), [+] 4mM L-glutamine, [+] 4500 mg/L glucose, [-] Sodium pyruvate, 5% FBS + 1% P/S. The concentration of calcium in DMEM is 1.8 mmol/L, which corresponds to a mild hypercalcemic environment (Beirman et al. 2012). While cell populations were expanding, media was changed every two days and passaged every four days. After sufficient expansion, ten wells of cells were seeded into 12-well plates with a count of about 0.5 x 10^6 cells/well. The treatment groups are described in Table 1. The pro-calcific media consisted of DMEM, 5% FBS, 1% P/S, 1.8 mM $CaCl_2$, 3.8 mM NaH_2PO_4, and 0.4 units (4ml) inorganic pyrophosphate (Rathan et al. 2014). Because the DMEM had 1.8 mmol/L of $CaCl_2$, the total calcium concentration in the pro-calcific media is 3.6 mmol/L (Dulbecco & Elkington 1975). The higher calcium and phosphate levels in this pro-calcific media have been demonstrated to be critical in inducing calcification in valve cells *in vivo* (Rathan et al. 2014). Blood serum calcium

content above 2.5 mmol/L corresponds to a severe hypercalcemic environment *in vivo* (Beirman et al. 2012). The inorganic phosphorous conditions correspond to a high serum phosphorous *in vivo* (Rubel & Milford 2003). The conditioned VEC media was used from each corresponding group. Each group was treated for seven days with one media change. The sample size was three groups of VICs corresponding to three separate populations of conditioned VECs.

Table 1. Treatment groups for rat VICs. Each group corresponds to a well.

Group	**Culture Media Content**
1	Regular Media
2	50% Regular Media + 50% Media from static-conditioned VECs
3	50% Regular Media + 50% Media from 0.0 OSI-conditioned VECs
4	50% Regular Media + 50% Media from 0.25 OSI-conditioned VECs
5	50% Regular Media + 50% Media from 0.50 OSI-conditioned VECs
6	Pro-calcific Media
7	50% Pro-Calcific Media + 50% Media from static-conditioned VECs
8	50% Pro-Calcific Media + 50% Media from 0.0 OSI-conditioned VECs
9	50% Pro-Calcific Media + 50% Media from 0.25 OSI-conditioned VECs
10	50% Pro-Calcific Media + 50% Media from 0.50 OSI-conditioned VECs

Following the treatment, the media was aspirated from the plates then washed with phosphate-buffered saline (PBS) to remove dead cells and remove media which might interfere with the next steps. Next, the cells were fixed to the plate with 10% formalin for ten minutes. The cells were then stained with Alizarin Red S, which binds to deposited calcium. The three plates were imaged and a heat map was generated using MATLAB (The MathWorks Inc., Natick, MA). First a color threshold was assigned and the brightness per pixel was determined. Then a random region of interest was chosen with the same location for each well in the plate, to reduce bias. Next the pixel intensity was measured, which corresponds to the dye concentration. The intensity values scaled from 1.0-0.0. The variation in color corresponds to concentration of

the dye. Red corresponds to 1 and is total coverage with calcium deposits. Blue corresponds to 0 which is very little coverage.

Statistical tests

All statistical analysis was done in SPSS 21 (IBM, Armonk, CA). A Shapiro-Wilk test for normality was performed for each level of the base media variable, regular, $W(15)= 0.94$ $p=0.387$, and pro-calcific media, $W(15)= 0.89$, $p=0.077$. A two-way ANOVA was used to compare the effect of the regular vs pro-calcific media, the treatment with VEC media and the interaction between the different variables. A post-hoc Tukey test was performed to compare the VICs treated in pro-calcific media and different treatments of VEC media. The level of significance was determined using an alpha value of 0.05.

RESULTS

After conditioning, the RNA was isolated from the cells and quantified prior to analyzing. The minimum amount of RNA per qPCR experiment is 100 ng/uL of RNA, all the samples had RNA concentrations considerably above the minimum. In order to determine the role of oscillatory flow on valve endothelial cells three genes associated with valve endothelial function were studied. Preliminary datum suggested that the group of cells treated with 0.5 OSI, the highest amount of flow reversal, show a substantially higher gene expression for van Willebrand factor (VWF) (Figure 2); there was moderately higher expression of elastin (ELN) and Notch homolog 1 (NOTCH 1). It is possible that the high oscillatory shear stress activated either disease or protective signaling mechanisms in the VECs.

Table 2. RNA quantification of rat valve endothelial cells (VEC) for each flow group, static, steady flow, 0.25 OSI and 0.5 OSI.

Rat VEC RNA Quantification

	Sample 1	Sample 2
Condition	RNA Concentration (ng/uL)	
Static	1381.5	547.65
Steady	1205.05	1397.55
0.25	1656	235.6
0.5	1320.9	622.65

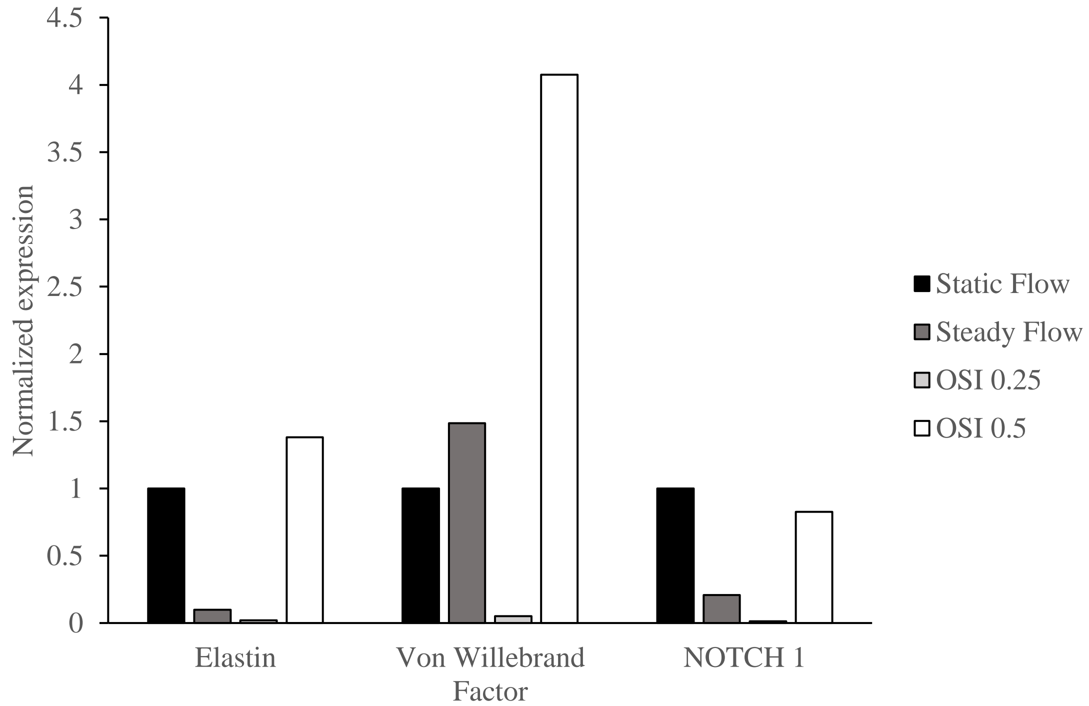

Figure 2. Very preliminary gene expression from one group (n=1) of rat valvular endothelial cells for elastin (ELN), van Willebrand factor (VWF), and Notch homolog 1 (NOTCH 1). Gene expression was represented for cells conditioned in each flow group: static, steady flow, 0.25 OSI and 0.50 OSI. The 0.50 treated VECs appear to have substantially higher upregulation for each gene analyzed.

The plates of treated rat VICs were stained with Alizarin red to quantify the amount of calcium deposition (Figure 3, Groups 1-10). A two-way ANOVA using the type of base media and conditioned VEC media as the independent variables showed that the type of base media made a significant difference in calcification ($F(1, 20) = 37.338$, $p = .0001$) and the type of conditioned VEC media made a significant difference in calcification ($F(4, 20) = 20.052$, $p = .0001$). In addition, the interaction between these variables was significant ($F(4, 20) = 27.58$, $p = .0001$).

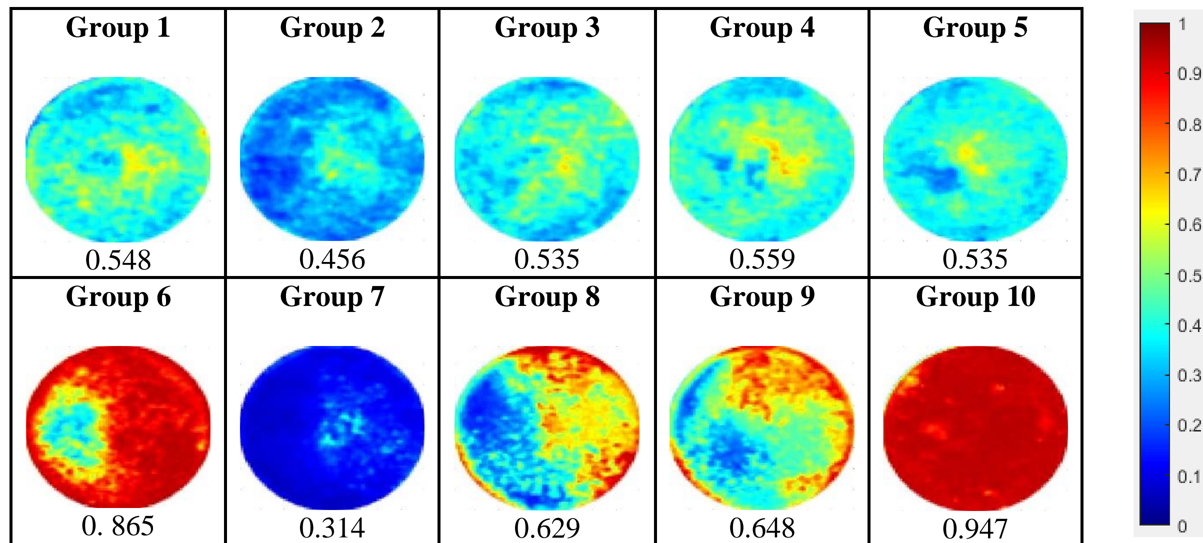

Figure 3. Rat valve interstitial cells (VIC) grown in various media treatments and stained to visualize calcium deposits on cell plates with corresponding heat maps. Heat map of alizarin dye corresponding to cell plates with raw intensity values below. The red in the heat map corresponds to a high degree of calcium deposits, while the blue corresponds to very little calcium deposits. Figure is representative of the mean signal intensity of all samples/group. Groups correspond to Table 1. Group 1, regular + no conditioned VEC media. Group 2, regular + static-conditioned VEC media. Group 3, regular + steady-flow-conditioned VEC media. Group 4, regular + 0.25 OSI-conditioned VEC media. Group 5, regular + 0.50 OSI-conditioned VEC media. Group 6, pro-calcific + no conditioned VEC media. Group 7, pro-calcific + static-conditioned VEC media. Group 8, pro-calcific + steady-flow-conditioned VEC media. Group 9, pro-calcific + 0.25 OSI-conditioned VEC media. Group 10, pro-calcific + 0.50 OSI-conditioned VEC media. Calcification is not significantly different in regular media treatment groups ($p>0.05$). In addition, group 10 had significantly higher calcium deposition than groups 7, 8, and 9 ($p<0.05$).

After a significant difference was demonstrated in the two-way ANOVA, a post-hoc Tukey test was conducted. Statistically significant differences ($p<0.05$) were only observed between groups (Groups 6-10; Fig. 3) that were cultured in pro-calcific media. Specifically, the control VICs with no conditioned VEC media were not statistically significant from the 0.50 conditioned VEC media ($p=0.92$). The VICs grown in 0.50 media had statistically significantly higher amounts of calcific deposits than the 0.25 OSI flow group ($p=0.02$), the steady flow group ($p<0.01$), and the static group. ($p<0.01$). The VICs treated with static-conditioned VEC media were statistically significantly lower in calcific deposits than all other groups ($p<0.01$). The VICs cultivated in the 0.25 OSI-conditioned media were not significantly different from the groups with no conditioned VEC media ($p= 0.13$) or steady flow-conditioned media ($p=0.98$). However, the steady flow group had significantly lower amounts of calcification than the VICs grown without VEC-conditioned media ($p=0.047$). These results may indicate that the process of calcification is dependent on both the presence of a high calcific environment and high oscillatory shear stress. It is possible that the VECs conditioned in steady flow and intermediate oscillatory flow, 0.25 OSI, released communication factors to the VICs signaling a decrease in calcific deposits.

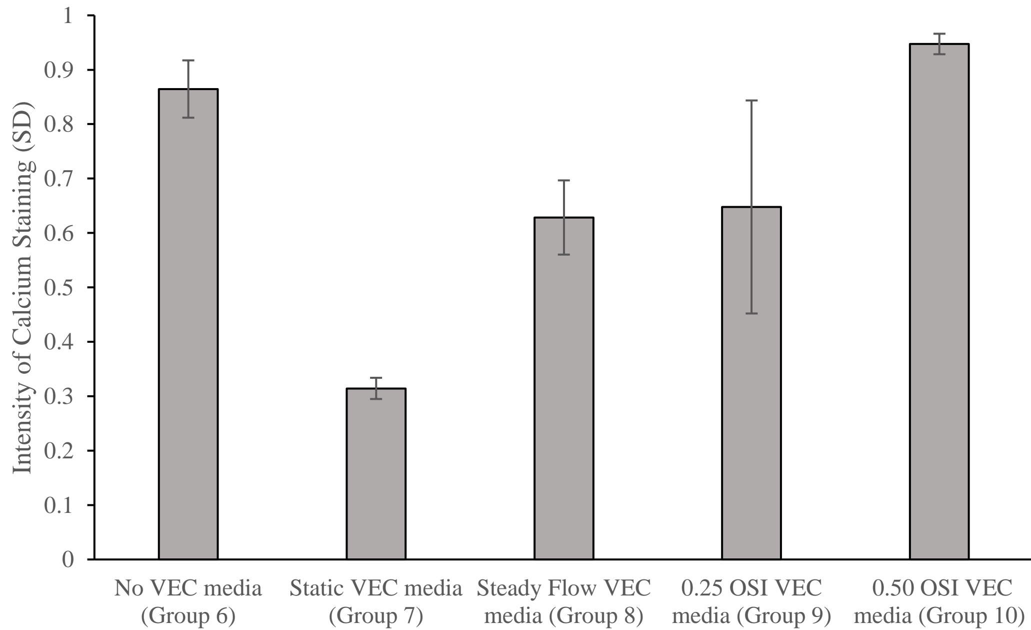

Figure 4. Rat valve interstitial cells (n=3). Group 6 was grown only in pro-calcific media and the rest were grown in 50% media from conditioned VEC group and 50% pro-calcific media. These results suggest that the VICs grown in 0.50-conditioned VEC media, group 10, got signals from the VECs to increase calcification.

DISCUSSION

Valvular interstitial cell dysfunction has been implicated in disease mechanisms. Calcium deposition in valve leaflets causes stiffening of the valve that leads to stenosis. Many factors may contribute to calcium deposition, such as substrate stiffness, fluid shear stress, tissue strain and biochemical cues (Hsu 2016). In this study, VIC cells were treated with extra biochemical stimulation by using pro-calcific media to induce calcification *in vitro*. A prospective study with patients between 40-79 years old found that high levels of serum phosphorous had significantly positive associations with the prevalence and incidence of aortic valve calcification (Hisamatsu et al. 2018). Another study found that patients with levels of serum phosphorous above 1.62 mmol/L were at risk for a surgical valve procedure (Rubel & Milford 2003). While no correlation was seen with levels of calcium above 2.63 mmol/L, patients with less than 2.2 mmol/L of calcium had less valve procedures (Rubel & Milford 2003). Conditioning of endothelial cells in steady flow and intermediate oscillatory flow appeared to create signals that stimulated the interstitial cells to not form calcium deposits. These signals may be protective against calcification in a pro-calcific environment. In contrast, the VEC media collected under high oscillatory flow conditions caused the highest amount of calcification in the interstitial cells grown in pro-calcific media, 3.6 mmol/L ionic calcium, which is suggestive of elevated blood calcium which may lead to higher risk of heart valve disease (Brown et al. 2017). In an environment of high calcium and phosphorous, as seen in severe aortic stenosis, high oscillations might contribute to interstitial cells calcification. Calcification of the heart valve is a complex, likely multifaceted process. In VIC-mediated valve calcification, VICs are activated to change phenotype to osteoblast-like bone forming cells and begin mineralization to form calcium nodules (Leopold 2012). Other processes that may be involved with promoting calcific valve

disease include infiltration of inflammatory cells, transition of endothelial cell phenotype, and differentiation of stem cells to osteoblast-like cells. In addition, matrix vesicles that sequester calcification inhibitors and acellular calcium deposits left by dead cells may play a role (Leopold 2012). However, interstitial cells are the most abundant cell types in valves and thus likely have the largest direct effect in valve calcification. The diminished protective capabilities of the VICs treated with high oscillatory-conditioned VEC media (OSI=0.50) might be resultant from a lowered production of endothelial paracrine signals that does not permit the downregulation of exogenous calcium and phosphate. A histological analysis into the nature of the calcific deposits could give insight into whether the calcium deposits were directly affecting the interstitial cells and able to develop into disease.

Endothelial cells receive environmental cues and send out signals to regulate the underlayer of interstitial cells (Chiu & Chien 2011). The aortic side of the valve leaflet is more prone to calcific lesions. There are several differences between the genetic profiles of endothelial cells on either side of the valve leaflet, which are exposed to different flow patterns. The VECs on the aortic side do not express as many inhibitors to calcification, however, the cells upregulate more genes related to protection against inflammation which is a precursor to calcification (Simmons et al. 2005). High oscillatory flow, as seen on the aortic side of the leaflet, could be compounded by an increase of serum phosphorous and calcium, often seen in the elderly, to stimulate calcification on the aortic side of the leaflet. While there seems to be some trends in the VEC gene expression data, the results are very preliminary. The VECs conditioned with 0.5 OSI, had higher van Willebrand factor (VWF), elastin (ELN) and notch 1 homolog (NOTCH 1) expression compared with the other flow types investigated. The protein VWF is an endothelial cells marker, which demonstrates that the phenotype was present (Armstrong & Bischoff 2004).

Increased expression of NOTCH 1 has been shown to have a role in inhibiting calcification through mechanisms that include downregulation of inflammation and structural remodeling to the calcific phenotype (White et al. 2015; Acharya et al. 2011). The higher NOTCH1 expression may be a protective response to inflammatory conditions. Further gene expression analysis on the remaining VECs samples will offer more concrete insights. Molecules with increased expression from the 0.50 OSI group could to be linked to upregulated gene expression in VICs to determine the mechanisms responsible for the increase of calcium deposits. These molecules could be used in targeted therapy in people with predisposition to calcification at areas of high oscillatory flow.

A limitation of this study was the shear stress magnitude used in the conditioning of the endothelial cells. While the averaged shear stress is physiologically relevant, *in vivo* the shear stress can range much higher in magnitude on either side of the leaflet. Since low shear stress is associated with disease, it could be a confounding factor. However, the substantial differences in calcific deposits between treated interstitial cells suggests the patterns of oscillatory flow had a significant effect on the response of the endothelial cells. The importance of VEC and VIC co-culture has been demonstrated by Butcher and Nerem (2006), as VECs have a critical role in regulating the VIC phenotype. However, valve endothelial cells are easily contaminated, over-populated and converted to interstitial cell types (Cheung et al. 2008). The effect of VEC regulation on VICs could potentially be transmitted through culture of VICs in VEC-conditioned media, rather than co-culture. In addition, cells exposed to the 0.50 OSI flow pattern in pro-calcifying media could be developed into a disease model that could be used to determine the mechanisms behind calcification and when testing possible treatments. In conclusion, high oscillatory shear stress (OSI= 0.50) combined with locally high phosphorous and calcium

concentrations could lead to paracrine (VECs-to-VICs) regulated mechanisms that increase valvular calcific disease. The identification of the molecular mechanisms responsible could lead to therapeutic discoveries for valve calcification.

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