Cyclic Aortic Pressure Affects the Biological Properties of Porcine Pulmonary Valve Leaflets

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Background and aim of the study: **Native pulmonary valve leaflets (PVL) are exposed to lower pressures compared to aortic valve leaflets. Knowledge of the biology of PVL exposed to aortic pressures is limited. Hence, the study's aim was to investigate the biological properties of PVL subjected to normal aortic pressures.**

Methods: **Porcine PVL were exposed to mean pulsatile pressures of 30 mmHg or 100 mmHg for 48 h in vitro. Subsequently, PVL were subjected to a mean pulsatile pressure of 30 mmHg for 48 h, followed by increased pressure (100 mmHg) for additional 48 h. Leaflets were evaluated by measuring collagen, DNA and sGAG contents in pressure-exposed and control PVL. Cusp morphology and cell phenotype were examined using hematoxylin and eosin staining (H&E) and alpha-smooth muscle actin (α-SMA) immunohistochemistry, respectively.**

Results: **PVL exposed to 30 mmHg showed no significant difference ($p > 0.05$) in collagen, DNA or sGAG contents compared to statically incubated PVL. However, PVL exposed to 100 mmHg showed a significant increase ($p < 0.05$) in both collagen and sGAG contents. Collagen content was also significantly increased ($p < 0.05$) in PVL exposed to varying pressures for 96 h compared to PVL exposed to 30 mmHg. The morphology of PVL exposed to cyclic pressures was comparable to that of both fresh and static leaflets, while α-SMA expression was decreased in PVL exposed to cyclic pressures when compared to fresh PVL.**

Conclusion: **PVL have the ability to withstand elevated mechanical conditions by increasing the total collagen and sGAG content of the leaflets. The structural integrity of the PVL is unaltered by changes in extracellular matrix composition. However, pulsatile pressures on the PVL did not preserve the native cell phenotype.**

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Aortic valve disease is the third most common cardiovascular disease in the United States (1), with the number of aortic valve replacements having steadily increased over the past 20 years (2). In 2002, a total of 93,000 heart valve replacement surgeries was performed in the United States alone (3). Current options for heart valve replacements include mechanical valves, bioprosthetic valves, and homografts. Patients receiving mechanical valves require long-term anticoagulation therapy in order to prevent thrombus formation (4-6), while bioprosthetic valves are usually not ideal substitutes in children as they have a high incidence of leaflet calcification and structural degeneration which would, in time, require reoperation (4,7). Consequently, bioprosthetic valves offer 10 to 20 years of reliable function before the need for reoperation (1,8). Autologous grafts that are implemented in the Ross procedure, are the best option for heart valve replacements in children (9,10) because of the graft's remodeling, growth, and reparative capabilities (10-12).

The Ross procedure is an established surgical procedure that utilizes the advantages of autologous grafts. In this procedure, the patient's diseased aortic valve is replaced with the patient's own pulmonary valve, and a cryopreserved cadaveric pulmonary valve is placed in the pulmonary valve position. Studies have indicated that Ross procedure has a 61% success rate and the patients survived as long as 26 years without reoperation (13). The high success rate of the procedure has led cardiologists to hypothesize that the pulmonary valve

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remodels to adapt to the aortic hemodynamic conditions. Additionally, human heart valves are known to be dynamic, sophisticated structures that interact closely with their surrounding environment, and any changes in the physiological environment can cause tissue remodeling on both micro and macroscopic scale (14,15).

Positioned between the right ventricle and the pulmonary artery, the pulmonary valve is exposed to a mechanically mild but complex environment, which includes fluid flow, cyclic pressure, shear and bending stresses, and cyclic flexure (15). Unlike the aortic valve, which is exposed to an average pressure load of 100 mmHg under normal resting conditions, the pulmonary valve is exposed to an average pressure load of 30 mmHg under resting conditions, but this may increase to 45 mmHg under hypertensive conditions. The tensile and compressive stresses generated by the hemodynamic conditions on the pulmonary valve are borne mainly by the loosely packed collagen, elastin and glycosaminoglycan (GAG) framework in the valve leaflets. Gerosa et al. (16) reported ultrastructural differences between pulmonary and aortic leaflets that include loosely packed collagen fibers in the fibrosa, thinner spongiosa and ventricularis layers in the pulmonary leaflets compared to the aortic leaflets. Due to these structural differences between the pulmonary leaflets and aortic leaflets, it is hypothesized that pulmonary leaflets adapt to the altered mechanical environment by changing the composition of their extracellular matrix (ECM).

The present studies focused on the biological response of porcine pulmonary leaflets to elevated pressure for different time periods. A custom-designed cyclic pressure system was used to examine the effects of aortic pressure on the biology of the pulmonary leaflets. Porcine pulmonary valves were chosen due to their anatomical and physiological similarities to the human pulmonary valve, and also their availability. The effects of pulsatile pressure were assessed by changes in collagen, DNA and sGAG contents, tissue morphology, and cell phenotype between the leaflets exposed to cyclic pressure and the control leaflets. Results from this study will improve our understanding of the behavior of the pulmonary valve leaflets following the Ross procedure.

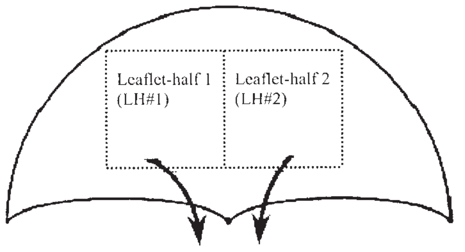
Materials and methods

Tissue harvest

Fresh porcine hearts were obtained from a local abattoir within 30 min of slaughter (Holifield's Farm, Covington, GA, USA). The pulmonary valves were excised on site and transported to the laboratory in sterile, ice-cold Dulbecco's phosphate-buffered saline (DPBS) (Sigma, St. Louis, MO, USA). Upon arrival at the laboratory, the three pulmonary leaflets were cut from the valve and the belly region of each leaflet was sectioned into two halves under sterile conditions (Fig. 1). Thirty pulmonary leaflets were divided into halves and subjected to the conditions of experimental group A (refer to nomenclature in Fig. 1). Likewise, 42 pulmonary leaflets were cut into halves and exposed to the experimental conditions of group B (see Fig. 1). In a subsequent set of experiments, 24 pulmonary leaflets were divided into halves and subjected to conditions that correspond to experimental group C (see Fig. 1). The pulmonary leaflets utilized in this study were randomized.

System set-up

The cyclic pressure system used in the current study was developed previously by Xing et al. (14). The valve leaflets were placed in a six-well tissue culture plates with each well containing 10 ml serum-free Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 50 mg/l ascorbic acid, 3.7 g/l sodium bicarbonate, 10 ml Non-Essential Amino Acid (Sigma), and 10 ml antibiotic-antimycotic solution



Experimental groups

LH#1	LH#2
A: Statically cultured, 48 h (STT48)	Pulsatile pressure of 45/15 mmHg, 48 h (PPT)
B: Statically cultured, 48 h (STH48)	Pulsatile pressure of 120/80 mmHg, 48 h (PPH)
C: Mean pulsatile pressure of 30 mmHg, 48 h (PPTN)	Mean pulsatile pressure at 30 mmHg, then pulsatile pressure increased to 100 mmHg for an additional 48 h (PPHN)

Figure 1: Preparation of pulmonary leaflets for the different experiments. A) One leaflet half was statically incubated and the other half cultured at a mean cyclic pressure of 30 mmHg for 48 h. B) Leaflet halves for the 48-h experiment at normal mean aortic pressure of 100 mmHg were prepared in a manner similar to those cultured at 30 mmHg. C) In the 96-h experiments, one half of the leaflet was exposed to a pulsatile pressure of 30 mmHg for 48 h; the other half was initially exposed to a pulsatile pressure of 30 mmHg for 48 h, then to pulsatile pressure of 100 mmHg for an additional 48 h.

(Sigma). The tissue culture plate was placed in a pressure chamber that was pressurized with 5% CO_2 and 95% air. Compressed air was used to achieve cyclic pressures by exerting a pulsatile force on a silicone membrane located at the top of the chamber. The pulmonary valve leaflets were exposed to a mean pulsatile pressure of 30 mmHg for 48 h (PPT), while leaflets statically incubated for 48 h under atmospheric pressure (STT48) were used as control (group A) (see Fig. 1). Leaflets were also subjected to a mean pulsatile aortic pressure of 100 mmHg for 48 h (PPH) and compared with statically incubated leaflets (STH48) (group B). Pulmonary leaflets initially exposed to a mean pulsatile pressure of 30 mmHg for 48 h and then subjected to mean pulsatile aortic pressure of 100 mmHg for an additional 48 h (PPHN) constituted experimental group C. Leaflets subjected to a mean pulsatile pressure of 30 mmHg for the initial 48 h of the 96-h experiments (PPTN) were used as controls. Fresh porcine PVL obtained from independent animals were used as controls for comparing hematoxylin and eosin (H&E) staining and α-smooth muscle actin (α-SMA) immunohistochemistry (IHC) of the PVL from all three experimental groups. The pressure chamber and control leaflets were placed in an incubator at 37ºC and 5% CO_2 atmosphere for the duration of the experiment. The effect of the pressure magnitude was examined at 30 ± 15 mmHg and 100 ± 20 mmHg.

Leaflet tissue processing

Following each experiment, the cultured leaflets from the pressure chamber and the static leaflets were cut into four strips. Three of the four strips were dehydrated for 48 h in a vacuum oven prior to digestion in pepsin (Sigma) or protease (Sigma) for the measurement of collagen, DNA, and sGAG content, respectively in the leaflets. The total amounts of collagen, DNA and sGAG were quantified using a Sircol Collagen™ assay kit (Bicolor, UK), Hoechst DNA assay, and Blyscan™ assay kit (Bicolor), respectively.

The fourth strip was used for qualitative analysis; tissue samples were fixed in 10% neutral buffered formalin for 24 h, saturated in 70% ethanol, then processed in a tissue processor (Shandon Pathcenter enclosed Tissue Processor), embedded in paraffin, and cut into 5-µm sections.

H&E staining was used to examine the morphological features of the leaflets. Staining was carried out using an automated slide stainer (Shandon Varistain XY Multi-Program Robotic Slide Stainer). The stained sections were viewed using a photomicroscope (Nikon 6000) equipped with a CCD digital camera (QImaging Retiga 1300C).

The presence of myofibroblast and smooth muscle cells in the tissue was indicated by α-SMA IHC. Following deparaffinization, the sections were blocked using 1% gelatin/PBS (Sigma) for 30 min. The slides were then incubated in mouse monoclonal anti α-SMA (Sigma) in 1% bovine serum albumin (BSA)/PBS for 1 h. The sections were saturated in biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) in 1% BSA/PBS, and 2% normal horse serum (Vector Laboratories) for 30 min. Avidin-D Texas red (Vector Laboratories) fluorochrome was applied to the sections and the cell nuclei were counterstained with 0.25 µg/ml DAPI (Sigma) and refrigerated at 4°C.

Total cell numbers in the tissue sections stained with H&E and α-SMA IHC were quantified using Image Pro plus software 4.0 (Media Cybernetics, Meyer Instruments, Houston, TX, USA). Cells from both the cultured and control leaflets were counted and expressed as total cell number for H&E-stained sections, while α-SMA cells were expressed as percentage population of positive cells.

Statistical analysis

For each experimental condition, quantitative data were normalized by dry tissue weight and expressed as mean ± SD. The Wilcoxon signed rank test (a non-parametric analysis) was used to assess the statistical significance of the data obtained on the collagen, sGAG and DNA contents. The test was also used to compare data acquired from counting H&E-stained cell nuclei and positive α-SMA cells between pressure-exposed PVL and their respective static control or PPTN leaflets. A Mann-Whitney non-parametric test was utilized to evaluate the statistical significance of data between: (i) fresh control and pressure-exposed PVL; and (ii) fresh and static control or PPTN PVL, as fresh control PVL were obtained from different animals. These non-parametric tests do not require a normally distributed population. A p-value <0.05 was considered to be statistically significant.

Results

No significant difference in collagen content was observed in PPT leaflets compared to STT48 leaflets (n = 30; Fig. 2). In contrast, PPH leaflets exhibited a 19.3% increase in collagen content compared to STH48 leaflets ($p < 0.05$, n = 42). PPHN leaflets showed a 43% increase in collagen content compared to PPTN leaflets ($p < 0.05$, n = 24). The collagen contents of the experimental groups are listed in Table I.

The sGAG content remained unchanged in PPT leaflets compared to STT48 leaflets ($p > 0.05$; n = 30). Similar behavior was observed in PPHN leaflets ($p > 0.05$; n = 24). However, in PPH leaflets, an increase in sGAG content was observed relative to STH48 leaflets ($p < 0.05$, n = 24). These data are shown graphically in Figure 3; actual values are listed in Table I.

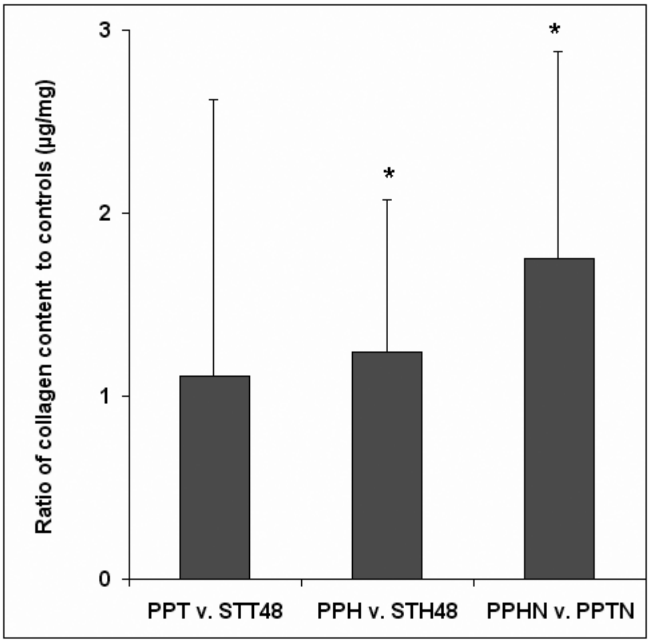


*Figure 2: Collagen content normalized by dry weight. Mean ± SD of PPT, PPH and PPHN leaflets relative to their controls. *Statistical significance (p <0.05): PPT v. STT48 (n = 30); PPH v. STH48 (n = 42); PPHN v. PPTN (n = 24).*

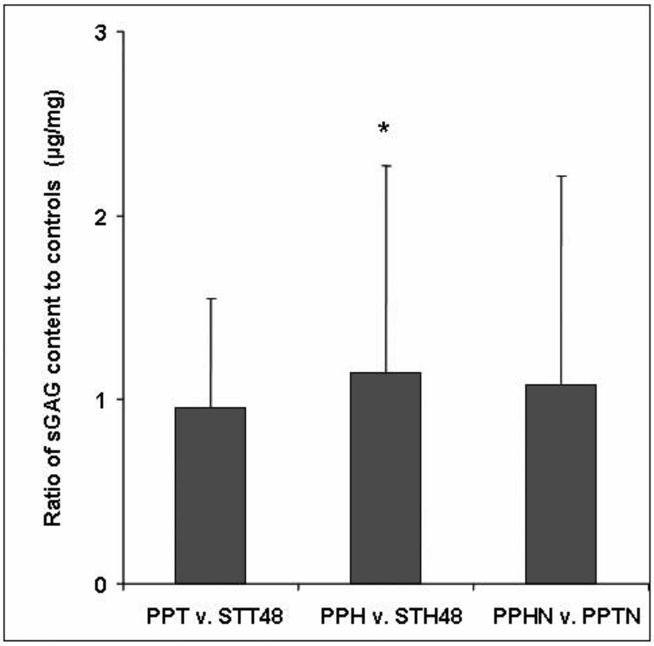


*Figure 3: sGAG content normalized by dry weight. Mean ± SD of PPT, PPH and PPHN leaflets relative to their controls. *Statistical significance (p <0.05): PPT v. STT48 (n = 30); PPH v. STH48 (n = 42); PPHN v. PPTN (n = 24).*

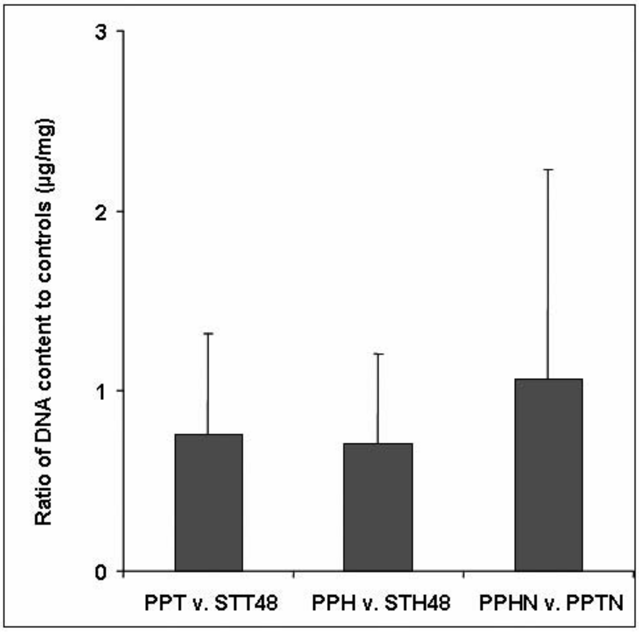


*Figure 4: DNA content normalized by dry weight. Mean ± SD of PPT, PPH and PPHN leaflets relative to their controls. *Statistical significance (p <0.05): PPT v. STT48 (n = 30); PPH v. STH48 (n = 42); PPHN v. PPTN (n = 24).*

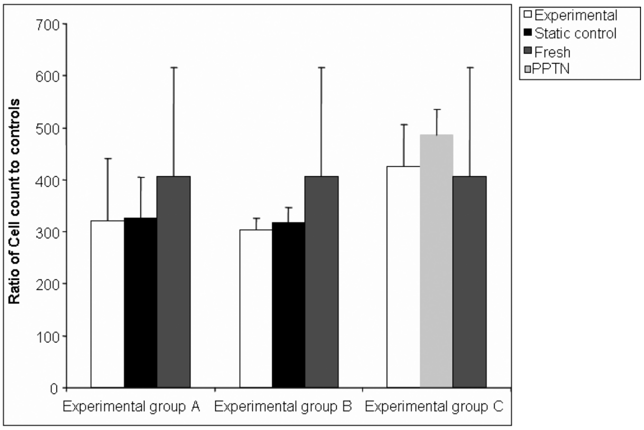


*Figure 6: Populations of cells (corresponding to eosin-stained nuclei) as a ratio of cells in the experimental leaflets to their respective controls (mean ± SD). *Statistical significance (p <0.05): PPT v. STT48 (n = 30); PPH v. STH48 (n = 42); PPHN v. PPTN (n = 24).*

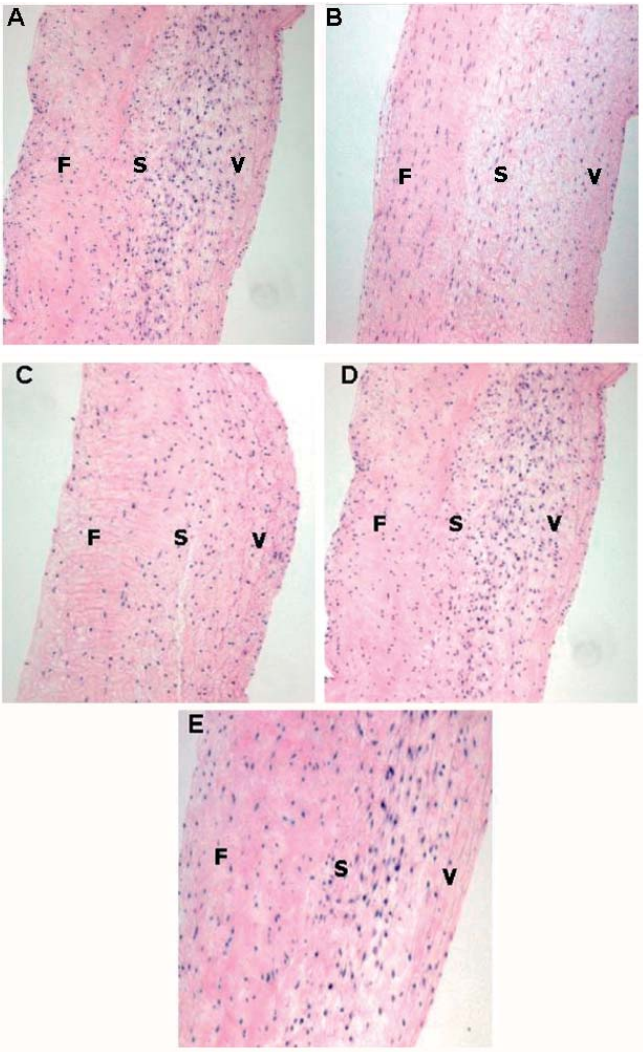


Figure 5: Hematoxylin and Eosin staining. A,B) Fresh and static control leaflets, respectively. C,D) PPT and PPH leaflets, respectively. E) PPHN leaflet. Pink and purple indicate the extracellular matrix and nuclei, respectively. F: Fibrosa; S: Spongiosa; V: Ventricularis.

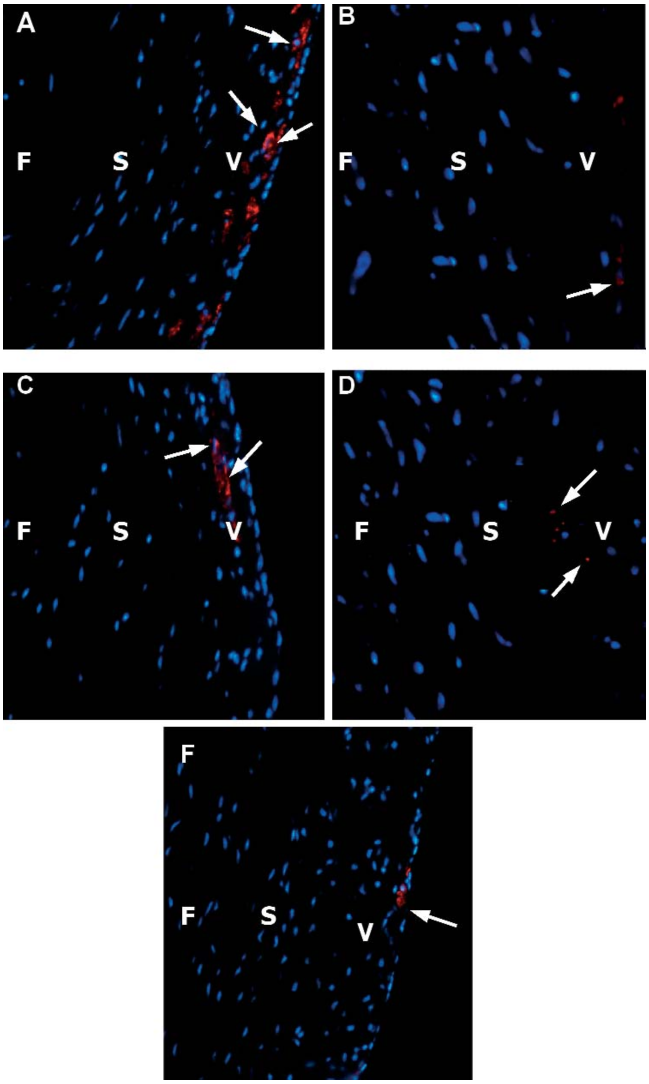


Figure 7: α-Smooth muscle actin staining. A,B) Fresh and static control leaflets, respectively. C,D) PPT and PPH leaflets, respectively. E) PPHN leaflet. α-Smooth muscle actin (α-SMA) was predominantly expressed in the ventricularis and spongiosa. Positive staining appears red; cell nuclei appear blue. F: Fibrosa; S: Spongiosa; V: Ventricularis.

Table I: Pressure experiment values: Collagen, sGAG and DNA contents normalized by dry weight (μg/mg).[*]

Pressure condition	Group A		Group B		Group C	
	PPT	STT48	PPH	STH48	PPHN	PPTN
Collagen (μg/mg)	8.28±4.54	7.44±1.51	18.6±7.67	15.0±9.23	25.6±17.1	14.6±10.5
sGAG (μg/mg)	85.0±25.1	89.4±41.9	114.0±41.9	100.0±37.1	40.9±24.8	39.0±21.8
DNA (μg/mg)	2.67±2.59	3.52±4.62	2.98±1.08	4.23±2.15	10.5±4.96	9.84±4.27

[*] Values are mean ± SD (n = 30 for 48-h experiment at 30 mmHg; n = 42 for 48-h experiment at 100 mmHg; n = 24 for 96-h experiments).

PPH: Mean pulsatile pressure 100 mmHg for 48 h; PPHN: Pulsatile pressure 30 mmHg for 48 h, then elevated pulsatile pressure 100 mmHg for an additional 48 h; PPT: Mean pulsatile pressure 30 mmHg for 48 h; PPTN: Pulsatile pressure 30 mmHg for the initial 48h of 96-h experiment; STH48: Statically cultured for 48 h. Controls for PPH leaflets; STT48: Statically cultured for 48 h. Controls for PPT leaflets.

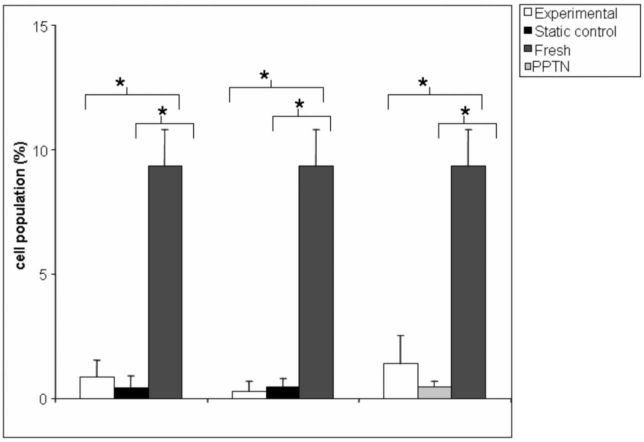


*Figure 8: Population of a-SMA cells as a percentage of the total cell population (mean ± SD). Data for experimental groups A, B, and C (see Fig. 1) indicated a significant decrease in cell population of experimental and static control leaflets relative to fresh leaflets. *Statistical significance (p <0.05).*

The DNA content in both PPT and PPH leaflets remained unchanged relative to their respective controls (Fig. 4) ($p > 0.05$, $n = 30$; $p > 0.05$, $n = 42$, respectively). This trend was also evident in PPHN leaflets ($p > 0.05$, $n = 24$).

H&E staining of the pulmonary leaflets exposed to different experimental conditions showed retention of the three-layered leaflet architecture compared to fresh and static leaflets (Fig. 5). No differences in leaflet structure or cell quantity ($p > 0.05$; Mann-Whitney and Wilcoxon signed tests; $n = 9$) were seen between the experimental leaflets from different pressure conditions and their respective control leaflets (Fig. 6). The results of α-SMA IHC indicated comparable α-SMA expression levels between PVL exposed to different experimental cyclic pressures and statically incubated PVL and PPTN (Figs. 7 and 8) ($p > 0.05$, $n = 9$). However, both the PVL exposed to cyclic pressure and statically incubated leaflets showed a significant decrease in α-SMA expression when compared to fresh PVL ($p < 0.05$, $n = 9$). No change was observed in α-SMA expression with increase in culture time for the PPHN leaflets when compared to PPTN or PPH leaflets.

Discussion

Native porcine PVL were exposed to a mean pulsatile pressure of 30 mmHg for 48 h, in vitro, to study the effects of normal physiological pressure load on the biological properties of the leaflets. From the results, it was concluded that the biosynthetic properties of pulmonary leaflets were not significantly altered by in-vitro dynamic culture of the leaflets at normal pulmonic pressures. The comparable collagen, sGAG and DNA contents between the pressure-exposed and static leaflets suggests that there is no loss of structural integrity in the leaflets. Based on the success rate of the Ross procedure, it was hypothesized that the pulmonary valve adapts to the aortic valve's hemodynamic environment. Results from the present study indicate that pulmonary leaflets exposed to aortic pressures for 48 h increased their collagen content, and this increase was also observed in PPHN leaflets. Collagen is the principal load-bearing structure of the leaflet, and an increase in its content is most likely the mechanism used by the PVL to adapt to harsher mechanical conditions. These results were comparable to the findings of Gaudino et al. (17), who detected a thicker fibrosa layer in hypertensive pulmonary valves compared to normal pulmonary valves, due to an increase in collagen synthesis. This positive correlation between collagen synthesis and pressure was also observed in studies by Xing et al. (14,15), who reported increased collagen synthesis in aortic valve leaflets in response to elevated pressure under both pulsatile and constant static pressure conditions. Furthermore, Luo et al. (18) observed an increase in type III collagen mRNA in lamina cribrosa cells after exposure to elevated hydrostatic pressure. Taken together, the results of these studies confirm that the elevation in collagen content is a potential mechanism for the PVL to adapt to elevated pressure both in vivo and in vitro.

The sGAG content was significantly greater in PPH leaflets than STH48 leaflets. After examining hypertensive rats, Hamada et al. (19) concluded that the increased mechanical stress and pressure contributed to an enhanced GAG synthesis observed in hypertensive rats. Additionally, Xing et al. (14,15) also noted an increase in sGAG content in aortic valve leaflets exposed to elevated cyclic pressure for 48 h. In contrast, PPHN leaflets did not exhibit the same trend as noted in the PPH leaflets and other reports. The present results indicated that sGAG content in PPHN leaflets is not affected by pressure when compared to PPTN leaflets. The results showed that sGAG synthesis is modulated by a greater increase in pressure load than required by the collagen content. A significant increase in sGAG content was observed in the pulmonary leaflets subjected to 100 mmHg mean pulsatile pressure when compared to static leaflets, but the increase was not significant compared to leaflets subjected to 30 mmHg pressure. This suggests that the increase in sGAG content is significant at a pressure difference of 100 mmHg between the PVL exposed to mean pressure of 100 mmHg and static PVL, while there is no statistical difference in sGAG content at a pressure difference of 70 mmHg between PVL exposed to mean cyclic pressures of 100 mmHg and 30 mmHg. Thus, it can be inferred that sGAG content increases with an increase in pressure magnitude, but the increase is significant only at pressure differences greater than or equal to 100 mmHg.

The primary cell types in pulmonary leaflets are endothelial and interstitial cells (20). Previous reports on the effect of elevated pressure on the DNA content have drawn multiple conclusions. Studies on the effects of elevated cyclic pressure on aortic valve leaflets indicated that endothelial cell proliferation is independent of elevated pressure (14), while another report noted that elevated hydrostatic pressure increases endothelial cell proliferation in bovine pulmonary arteries (21). The present results indicate that the DNA content of pulmonary leaflets exposed to different pulsatile pressures are comparable to those of the respective controls. The insignificant changes in the DNA content can be attributed to a combination of the inhibited endothelial cell proliferation and the concurrent interstitial cell proliferation, as suggested by Hishikawa et al. (22) and Xing et al. (15). Details of the true mechanism remain unknown, and further investigation on the effects of pressure on endothelial cells is needed. The results obtained from the study of DNA content in pulmonary leaflets suggest that the preferred mechanism for the adaptation of pulmonary leaflets cells to elevated pressures is by the up-regulation of leaflet collagen and sGAG content, and not by increase in cell number.

H&E staining showed the retention of the normal morphology and cell density of the leaflets. The results from the present study were in agreement with those presented by Rabkin-Aikawa et al. (23), who showed that pulmonary autograft valves demonstrated preserved pulmonary leaflet morphology after exposure to aortic valve hemodynamics, in vivo. This observation indicates that cyclic pressure forces do not alter the morphological features of the leaflets. Quantitative analysis demonstrated a comparable number of cells in the fresh, statically incubated and leaflets cultured under cyclic pressures. However, α-SMA expression was decreased in both pressure-exposed and static leaflets when compared to native levels. This suggests that physiological cyclic pressures alone do not retain the native cell phenotype of the pulmonary leaflets. Several studies, including that by Weston et al. (24), have shown this decrease in α-SMA expression following the exposure of valvular leaflets to isolated effects of fluid flow and steady shear stresses (14,15). Additionally, Yperman et al. (25) showed that ovine valvular interstitial cells (VIC), when cultured under static conditions, undergo phenotypic modulation to activated myofibroblasts while retaining a stable ECM. Observations by Xing et al. (14,15) on the effect of elevated constant and pulsatile pressures on cell phenotype of aortic leaflets are consistent with those of the present study. In-vitro culture under the influence of isolated cyclic pressures appears to have caused changes in the polymerization/depolymerization equilibrium of F-actin and G-actin of smooth muscle cells in pulmonary leaflets. In fact, the decrease in the α-SMA from the native level suggests that F-actin depolymerization was favored over its synthesis due to the absence of leaflet contractility in vitro.

In conclusion, the results of the present study showed that at aortic pressures, the pulmonary valve leaflets adapt to changes in mechanical conditions by increasing their collagen and sGAG content within 48 h. The insignificant difference in DNA content under all experimental conditions implied that the existing cells up-regulated the synthesis of both collagen and sGAG during the 48-h period at aortic pressures. In the 96-h studies, however, the different responses of sGAG and collagen to pressure conditions indicated that sGAG and collagen require different pressure thresholds before any noticeable change occurs in their contents. Additionally, cyclic pressure forces do not preserve the native cell phenotype of the valve leaflets.

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