Pregnancy-Induced Remodelling of Heart Valves

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ABSTRACT

Recent studies have demonstrated remodelling of aortic and mitral valves leaflets under the volume loading and cardiac expansion of pregnancy. Those valves’ leaflets enlarge, with altered collagen fiber architecture, content and crosslinking and biphasic changes (decreases, then increases) in extensibility during gestation. This study extends our analyses to right-sided valves, with additional compositional measurements for all valves. Valve leaflets were harvested from non-pregnant heifers and pregnant cows. Leaflet structure was characterized by leaflet dimensions, and ECM composition was determined using standard biochemical assays. Histological studies assessed changes in cellular and ECM components. Leaflet mechanical properties were assessed using equibiaxial mechanical testing. Collagen thermal stability and crosslinking were assessed using denaturation and hydrothermal isometric tension tests. Pulmonary and tricuspid leaflet areas increased during pregnancy by 35% and 55% respectively. Leaflet thickness increased by 20%: only in the pulmonary valve and largely in the fibrosa (30% thickening). Collagen crimp length was reduced in both the tricuspid (61%) and pulmonary (42%) valves with loss of crimped area in the pulmonary valve. Thermomechanics showed decreased collagen thermal stability with surprisingly maintained crosslink maturity. The pulmonary leaflet exhibited the biphasic change in extensibility seen in left side valves, while the tricuspid leaflet mechanics remained largely unchanged throughout pregnancy. The tricuspid valve exhibits a remodelling response during pregnancy that is significantly diminished from the other three valves. All valves of the heart remodel in pregnancy, in a manner distinct from cardiac pathology, with much similarity valve-to-valve, but with interesting valve-specific responses in the aortic and tricuspid valves.

NEW & NOTEWORTHY

This work shows, for the first time, all the valves of the maternal heart remodel in response to the volume overload and other cues associated with pregnancy. This remodelling response is not subtle, but produces great changes in valve size, ECM architecture, and biomechanics. Such fundamental insight presages valve therapy.

INTRODUCTION

Heart valves are complex and dynamic biological structures. They play important roles toward efficient cardiac pumping, ensuring unidirectional blood flow while opening and closing 3 billion times in a lifetime. The extent and the mechanisms via which the valves may adapt to changing loading conditions, in either physiology or pathology, remain unclear. Many investigations of valve adaptation have focused on physiological development (8, 20, 51-53) or pathological conditions such as left ventricular (LV)
dysfunction (10), mitral valve regurgitation (58), myxomatous degeneration (19), or heart failure (17).

Recent studies from our laboratory, however, have demonstrated the capacity of maternal heart valve leaflets to remodel under the altered hemodynamics of pregnancy: a non-pathological condition (38, 39, 60). Pregnancy is accompanied by an increase in blood volume and cardiac output of ~50%, as the maternal circulation accommodates the developing feto-placental unit. This is volume overload produces cardiac enlargement (21, 45, 60) with expansion of valve orifices (areas increasing from 12-53% in humans (9, 43)). These changes in geometry then elevate stresses on closed valve leaflets per the Law of Laplace (26). Our studies of the aortic and mitral valves have thus far shown dramatic increases in leaflet size of both valves, accompanied by alterations in fiber architecture and composition, a decrease in thermal stability of collagen, a reversible shift in leaflet extensibility, and valve-specific alterations in resistance to creep (38, 39, 60). Together, these results provide strong evidence of both collagen turnover during pregnancy and unexpected maturation of native leaflet collagen: a complex restructuring of the collagen matrix under the altered cardiovascular loading and hormonal shifts of pregnancy.

While remodelling in pregnancy has been described in the aortic and mitral valves (39), it is important to examine all four valves since their responses may differ due to differences in their (i) embryological origin or (ii) mechanical loading conditions. For instance, the inflow valves (mitral and tricuspid) arise solely from tissues of the endocardial cushions in the developing heart tube, while the outflow valves (aortic and pulmonary) are also derived from immigrating neural crest cells (5, 12, 20, 22, 47). On the other hand, higher transvalvular pressures (TVP) in the left side of the heart result in higher tensile stresses in the mitral and aortic valve leaflets (3, 32). The remodelling response to pregnancy may therefore be valve-specific. Indeed, valvular regurgitation is predominantly right-sided in pregnant women. While 95% of pregnant women experience regurgitation on their right side (low pressure) pulmonary and tricuspid valves, only 27% experience regurgitant flow on the mitral and none in the aortic valve (9). One explanation for this pattern is that only the left-sided valve leaflets remodel more during pregnancy, and thus can better-maintain coaptation.

There is indeed some evidence to suggest that (i) the state of the collagen matrix (thermal stability and turnover), and (ii) valvular interstitial cell (VIC) phenotype and activity are valve-specific, perhaps influenced by the embryological origin and/or the loading conditions of the tissue. For instance, Aldous et al. found that, valve-to-valve, collagen denaturation temperature was inversely correlated with increasing transvalvular pressure (3). Further, tissue from inflow valves contained significantly less mature and total crosslinks compared to outflow valves. This implied that the inflow valves had less mature collagen, due perhaps to faster remodelling than occurred in the outflow valves (3). Studies from Merryman et al. have correlated VIC phenotype and TVP. VICs from the left-sided valves (higher TVP): (i) were significantly
stiffer (31, 32), (ii) contained higher contents of heat shock protein 47 (Hsp47, a collagen synthetic protein) (32), (iii) contained more alpha smooth muscle actin (31), α-SMA, a cytoskeletal protein marker of the “activated” VIC phenotype (7, 30)), and (iv) showed increased contractility (31). Together, these studies suggest that valves from the higher-pressure left side of the heart have VICs in the activated, synthetic state that increase the “remodelling potential” of the tissue. Whether such potential plays out during pregnancy-induced remodelling is unknown.

Based on the knowledge in hand, we hypothesized that the valves of the right sided of the heart would exhibit a decreased remodelling response to pregnancy, this in comparison to the striking response previously seen in the left-side mitral and aortic valves. To test this hypothesis, we have continued to use our bovine pregnancy model which closely emulates the features of human pregnancy. For the work described herein, we applied the same multidisciplinary combination of dimension measurements, biaxial mechanical testing, histology, thermoelastic studies, and biochemical analysis to the right side valves which we had previously used in our studies of the left side valves (38, 39, 60). We also added new assessments of matrix composition (water, elastin, and sulphated GAG contents), cell counting, and assessment of cell phenotype to samples of all four valves: in both non-pregnant and pregnant animals.

The data from this study, in combination with our previous results, paint an intriguing picture of extensive valvular remodelling during the physiological process which is pregnancy.

**GLOSSARY**

\[ \lambda_{\text{peak}}^C \]: circumferential stretch ratio under peak (60 N/m) equibiaxial tension (circumferential extensibility)

\[ \lambda_{\text{peak}}^R \]: radial stretch ratio under peak (60 N/m) equibiaxial tension (radial extensibility)

Areal Stretch: \( (\lambda_{\text{peak}}^C \times \lambda_{\text{peak}}^R) \), areal stretch under peak (60 N/m) equibiaxial tension (net tissue extensibility)

\( T_d \): denaturation temperature

t\(_{1/2}\) control: HIT half-time of load decay: index of mature, thermally stable collagen crosslinking

t\(_{1/2}\) treated: HIT half-time of load decay following NaBH\(_4\)-treatment: index of total collagen crosslinking (immature + mature)

Crosslink Index: \( (t_{1/2}\text{treated}/t_{1/2}\text{control}) \), index of immature collagen crosslinking
METHODS

Tissue Harvest and Sample Preparation

Protocols for the harvesting of bovine tissues were approved by the University Committee on Laboratory Animals (UCLA) at Dalhousie University. Bovine hearts were purchased as food from a local abattoir (Armstrong Food Services Limited, Kingston, Nova Scotia, Canada) immediately following slaughter. Hearts were collected from heifers (sexually mature, never-pregnant female cattle), and from pregnant cows. To assess gestational age in pregnant cattle, fetal crown-rump length was measured, with a full term (278-290 days gestation) bovine fetus measuring approximately 100 cm (14). Pregnant animals ranged in gestational age from 61 days (9 cm fetal length) to 256 days (87 cm fetal length). Due to imposed federal food regulations at Canadian Abattoirs, all cattle were under 30 months of age and did not exhibit signs of illness (Canadian Food Inspection Agency, CFIA).

Due to structural consistency between aortic and pulmonary valve leaflets, all three leaflets of these valves were excised. Since the mitral and tricuspid valves possess significant structural and anatomic differences between leaflets, all experiments used the larger anterior (mitral) and posterior (tricuspid) leaflets. The mitral valve anterior leaflet experiences higher in vivo stresses as compared to the posterior leaflet (24, 25). There is, however, no evidence to suggest that the tricuspid valve leaflets differ in mechanical loading conditions. Still, to ensure consistency with previous studies (3), the larger posterior leaflet was used. All leaflets were excised as close to the valve root as possible. Leaflets were washed with Hanks’ physiological solution (pH 7.4) including 6 mg/l trypsin inhibitor (lyophilosate), and an antibiotic-antimycotic agent and containing 10,000 units/ml of Penicillin G, 10 mg/ml streptomycin sulphate, and 25 µg/ml amphotericin B (Sigma-Aldrich Canada, Oakville, ON, Canada).

Mechanical extensibility, dimensions, crimp length, total collagen content, and thermal properties of leaflets from the aortic and mitral valve were presented in our previous studies (39, 60) and are extended here to include the pulmonary and tricuspid valves.

Mechanical Extensibility Testing

Pulmonary and tricuspid valve leaflets from 9 non-pregnant heifers, and 13 pregnant cows, were excised and a 1cm-square specimen was cut from the center belly region of each leaflet (39, 60) for biaxial mechanical testing (MTS, 458 Series, Eden Prairie, MN). Biaxial deformation of the sample was measured by optical tracking of a 2 by 2 grid of four small adherent graphite markers. Specimens were extended biaxially (in circumferential and radial directions) to a peak equibiaxial tension of 60-N/m, and axial deformations were obtained via marker tracking using Image J (National Institutes of Health). Axial
stretch ratios (extensibilities): $\lambda_{C}^{\text{peak}}$ (circumferential extensibility) and $\lambda_{R}^{\text{peak}}$ (radial extensibility) under peak equibiaxial membrane tension (60-N/m). The net tissue extensibility was represented by the areal stretch under peak equibiaxial tension and was calculated using the following equation:

$$\text{Areal Stretch} = (\lambda_{C}^{\text{peak}} \times \lambda_{R}^{\text{peak}})$$

Finally, tension versus areal stretch graphs were plotted using areal stretch values calculated at equibiaxial tensions of 1.0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, and 60.0-N/m, to assess leaflet tissue properties over the entire loading range examined under biaxial loading.

Leaflet Dimensions

Fresh, excised aortic and mitral valve leaflets from 10 non-pregnant heifers and 15 pregnant cows, were laid flat and photographed (Nikon D50 camera, Nikon, Tokyo, Japan) with a reference scale bar. Images were imported into image-analysis software (Image J, National Institutes of Health), where radial midline length and circumferential midline length were measured, and the freehand selection tool was used to outline the perimeter of the leaflet to calculate the total area of the leaflet (39, 60).

Biochemical Analysis

Following imaging for leaflet dimensions, the same group of valves (from 10 non-pregnant heifers and 15 pregnant cows) was prepared for biochemical assays for total and acid-soluble collagen, glycosaminoglycans and elastin. Heart valve leaflets for all assays were wrapped in cheesecloth soaked in Hanks’ physiological solution, placed immediately in the -86°C freezer, and stored until they could be assayed. The first set of frozen leaflets was freeze-dried for a minimum of 52 h. Dry samples of approximately 10 mg dry weight, from the belly region of the leaflet, were weighed. Their total collagen content (normalized to dry weight) was estimated from the hydroxyproline content (23) following the protocol of Woessner (61).

Biocolor biochemical assay kits (Biocolor Ltd., Carrickfergus, UK : Accurate Chemical & Scientific Corporation, Westbury, NY, USA) were used to assess the content of sulphated glycosaminoglycans (sGAG; Blyscan Assay), elastin (Fastin Assay), and acid- and pepsin-soluble collagen (Sircol Assay). This extractable form of collagen is often interpreted to be newly laid down collagen that is not sufficiently crosslinked into the network to resist solubilisation. For these studies, the second set of frozen leaflets was thawed and dissected to take samples from the belly of the leaflet, which were weighed prior to testing. All samples were assayed in duplicate and compared to a set of blanks and standards. Following the assay, a microplate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, Vermont) was used to measure the
dye absorbance of each prepared plate at a specified wavelength (656 nm : Blyscan, 513 nm : Fastin, and 555 nm : Sircol). Dry weight measures of glycosaminoglycans, elastin, and extractable collagen were calculated using the wet/dry weights from each valve.

**Histological Analysis**

Leaflet histology from aortic and mitral valves was presented in our previous study (39). In the present study, we extend these analyses to include the pulmonary and tricuspid valves, with the addition of immunohistochemical assessment of VICs in all four valves.

Leaflets were collected from another set of animals (9 non-pregnant heifers and 13 pregnant cows) and prepared for histological analysis as described in our earlier studies (38, 39). Each leaflet was divided to produce two symmetric halves (Fig. 2) that were fixed (10% neutral buffered formalin), embedded, and sectioned into 5μm serial sections. Circumferential cross-sections were cut from one half of the leaflet for picrosirius red staining to examine collagen alignment and crimp. Radial cross-sections were cut from the other half of the leaflet for Verhoeff-Van Gieson staining to identify leaflet layering (Verhoeff-Van Gieson Elastin Staining Kit, Polysciences, Inc., Washington, PA) as well as immunohistochemical (IHC) staining of the activated VIC phenotype marker alpha-smooth muscle actin (α-SMA). Each radial section contained the complete leaflet cross-section from the free edge to the fixed edge.

**Collagen Crimp Analysis**

Images were taken of picrosirius-strained sections using a Nikon Eclipse E600 light microscope equipped with a polarizer and a 10MP AmScope digital camera. As described in our previous studies (38, 39), for both measures, six contiguous images were taken at 40x objective magnification along the circumferential direction, spanning 2 mm into the belly region of the leaflet, and analyzed using Image J software. Collagen crimp was characterized by (i) crimp length, (peak-to-peak measurement of one crimp period) and (ii) percentage of the leaflet area which was crimped. Both crimp length and crimped area were averaged across all 6 images for each valve.

**Leaflet and Layer Thicknesses**

As previously described (38, 39), Verhoeff-Van Gieson stained sections were photographed at low magnification along the entire length of the valve leaflet (Zeiss Axioplan 2 Imaging Microscope with AxioVision software (Release 4.8.1) and images from each valve were assembled into a single mosaic using Image J with plugins Mosaic J (57) and TurboReg (56). Thickness measurements, of the full leaflet as well as of each layer (fibrosa, spongiosa, ventricularis (tricuspid only) or atrialis (pulmonary only)), were taken at 25 equidistant vertical lines that transected the full thickness of the tissue along the length of
the leaflet. Thickness measurements were then averaged across the length of the leaflet for each valve sample.

Cell Density and Phenotype

Radial leaflet sections were also used for immunohistochemistry (IHC) staining of cytoplasmic alpha-smooth muscle actin (α-SMA) to identify alterations to cell phenotype associated with pregnancy, using the standard IHC protocol detailed by Ramos-Vara et al. (42), and explained here briefly. Slides were deparaffinised, rehydrated, and pretreated with heat-induced epitope retrieval (HIER). This antigen retrieval process modifies the molecular conformation of proteins by removing protein crosslinking caused by aldehyde-based fixatives (40, 42, 54), improving subsequent IHC staining. Tris-ethylenediaminetetraacetic acid (EDTA) buffer (pH 9) was used in the HIER process. To reduce non-specific background staining, endogenous peroxidases were blocked using hydrogen peroxide and endogenous biotin protein was blocked with avidin/biotin protein block. Incubation of the primary antibody (monoclonal mouse anti-human smooth muscle actin, M0851, Dako, Glostrup, Denmark) was performed in humid chambers at 37 °C overnight. The secondary biotinylated link antibody (Dako) was applied, followed by the application of streptavidin-peroxidase conjugate (horseradish peroxidase – HRP) to bind to biotin for signal amplification. Diaminobenzidine-HRP (DAB) was then used to allow for visualization of streptavidin-HRP, and finally slides were counterstained with Mayer’s hematoxylin solution and Scott’s water, dehydrated, and mounted with glass cover slips. In parallel with each staining group, we included with a negative control (no primary antibody), an isotype control (Mouse IgG2a, X0943, Dako), and a positive control. Antibody optimization for stain quantity and intensity was performed prior to the staining of all leaflet tissues and was determined to be at a dilution of 1:200 for both the primary α-SMA antibody as well as the IgG2a isotype control.

Digital images were taken across the thickness of the valve leaflet at 40x objective magnification (Zeiss Axioplan 2 Imaging Microscope and imaging software (AxioVision, Release 4.8.1). Cell counting was performed using ImageJ imaging software Count Cell plugin and two counter types: Type 1 counts all nuclei (total cell number); Type 2 counts the nuclei from α-SMA-positive cells (α-SMA+ cell number) (Fig. 1b). The percentage of α-SMA-positive cells (% α-SMA+) was calculated from the ratio of positive stained cells to total cell count.

Denaturation Temperature Testing and Hydrothermal Isometric Tension Testing

Collagen thermal stability and crosslinking were presented in our previous study for the aortic and mitral valve (39) using denaturation temperature testing (DTT) and hydrothermal isometric tension (HIT) tests
The present study extended these analyses to include the pulmonary and tricuspid valves collected from another set of animals (10 non-pregnant heifers and 23 pregnant cows). Briefly, circumferential strips cut from the belly region were mounted under isometric tension in a bath of distilled water. The denaturation temperature, $T_d$, is indicated by a sharp increase in tension (4, 62) as the bath temperature is increased from ~20°C to 90°C. Following the heating segment, a 90°C isotherm was maintained for three hours, during which the isometric tension is largely supported by the denatured collagen in the sample. The tension decays as primary peptide bonds of the collagen network hydrolyse, allowing slippage of the chain fragments (28). The rate of relaxation is independent of its non-collagenous constituents and is closely correlated to the concentration of thermally stable collagen crosslinks (4, 27): the more crosslinks present, the slower the relaxation rate. The tension relaxation half-time ($t_{1/2}$ control) thus provides an indicator of mature collagen crosslinking. An index of total collagen crosslinking ($t_{1/2}$ treated) was obtained from the HIT $t_{1/2}$ of paired tissue samples after immature crosslinks were reduced to a heat-stable form with NaBH4 (59), with the ratio $t_{1/2}$ treated / $t_{1/2}$ control provides an immature “crosslinking index”.

**Statistical Analysis**

All results are expressed as the mean ± standard error of the mean (SEM), with the n value representing the number of animals per group. All data were identified by the valve from which leaflet tissue was harvested and by two anatomical locations (inflow and outflow), by sidedness (left side and right side) and by the two pregnancy states: NP (non-pregnant) and P (pregnant). Results and regressions were considered significant for $p < 0.05$. All statistical testing was carried out using JMP 11 software (SAS).

For examination of new data from right side valves only, a 2-way ANOVA (MANOVA) was applied with variables of valve type (pulmonary, tricuspid) and pregnancy state (NP, P). Where a variable was identified as having a significant effect, a two-tailed t-test was applied (two groups only).

For data on individual right side valves, the effect of gestation time was assessed for the pregnant group by dividing the data into EP (early pregnant) and LP (late pregnant) groups according to fetal crown-to-rump length, in accordance with our previous study (39). Cows carrying fetuses of crown-to-rump length < 45 cm (0–169 days) were classified as EP, and those carrying fetuses > 55 cm (193–270 days) were classified as LP. The mid-pregnancy group of 45–55 cm (169–193 days) was omitted in this comparison to obtain a more complete separation of early and late groups. A one-way ANOVA was performed, followed by Tukey honestly significant difference comparisons among the three groups (NP, EP, and LP). To evaluate changes in any parameter as a continuous function of pregnancy duration, data were also plotted as a function of fetal length and fitted with a least-squares linear regression.
Where new data was collected from all 4 heart valves (biochemical composition and cell studies), or available for all four valves via comparison with our previous work (39), comparisons were made between valves of differing anatomical positions: (i) left-side (aortic and mitral) valves vs. right-side (pulmonary and tricuspid) valves to reveal the effects of loading conditions on the remodelling response; (ii) inflow (mitral and tricuspid) valves vs. outflow (aortic and pulmonary) valves to reveal the effects of embryological origin on the remodelling response; as well as (iii) individual valve types (aortic, mitral, pulmonary, and tricuspid independently), to uncover the presence of valve-specific remodelling. In each of these three cases, analysis began with a two-way ANOVA (MANOVA) with variables of anatomical location (inflow/outflow, left-side/right-side, or valve type) and pregnancy state (NP, P). Where the MANOVA showed significant effects of a variable, one-way ANOVA was performed with respect to that variable, followed by Tukey’s honestly significant difference comparisons. In the rare case where the MANOVA showed a significant interaction term, then groups were separated appropriately and analyzed individually.

RESULTS

For the present paper, new data have been generated for the right side bovine pulmonary and tricuspid valves, from pregnant and non-pregnant animals, using the same methods which we previously applied in our isolated study of the left side aortic and mitral valves (39). These new data are presented below and, where appropriate, briefly set in the context of the previous data. Some new methods (water, collagen, and sGAG contents, cellularity, and immunostaining for cell phenotype) have also been applied to new specimens from all four valves, again in both pregnant and non-pregnant cattle. These data are presented for all four valves.

Leaflet Mechanical Properties

Peak areal stretch at 60-N/m equibiaxial tension (net extensibility) in the pulmonary valve decreased 14% (Table 1), during early pregnancy, then increased linearly during fetal growth (Fig. 2), returning to pre-pregnant values by late pregnancy (Table 1). Similar, biphasic changes in extensibility had previously been observed in the aortic and mitral valves (39). The tricuspid valve, however, was quite different. There was no significant change in net leaflet extensibility during pregnancy, despite a slight, reversible decrease in circumferential extensibility in early pregnancy (Table 1). Thus, changes in leaflet extensibility during pregnancy were largely in the radial direction for the pulmonary valve, but in the circumferential direction for the tricuspid valve (Table 1).
Leaflet extensibility was also assessed at several levels over the range of equibiaxial tensions examined in our mechanical loading experiment. In the pulmonary valves, at each tension above 5-N/m, peak areal stretch decreased with early pregnancy (leftward shift of the tension stretch curve), and then increased in late pregnancy: a reversible decrease in leaflet extensibility (Fig. 3A). This same behavior was also observed in the aortic and mitral valves, presented in our previous study (39). In the tricuspid valve, mean areal stretch at each tension decreased from non-pregnant to early pregnant animals (leftward shift of curve), and then remained unchanged in late pregnancy: an irreversible decrease in leaflet extensibility (Fig. 3B).

Leaflet Dimensions

Leaflets from both right-sided valves underwent significant increases in area during pregnancy, increasing in both their circumferential and radial lengths (Table 2). These enlargements are again in agreement with the pregnancy-induced changes observed in the left-sided valves (39)). Mean leaflet area increased by 35% and 55% in the pulmonary and tricuspid valves respectively, contributed to by 19-24% increases in radial length and 19-21% increases in circumferential length across the valves.

Leaflet and Layer Thicknesses
Thickness of the tricuspid valve leaflet was unchanged during pregnancy (Table 3)—as had been observed in the mitral and aortic valves (39). In the pulmonary valve, total leaflet thickness increased 20% between non-pregnant and pregnant animals, with a 31% increase in the fibrosa thickness (Table 3). While fibrosal thickening during pregnancy was also seen in the aortic and mitral valves (39), it did not occur in the tricuspid valve (Table 3).

<table>
<thead>
<tr>
<th>Table 3. Changes in leaflet thickness and individual layer thicknesses</th>
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<tr>
<td>Thickness, μm</td>
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<td>----------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Fibrosa</td>
</tr>
<tr>
<td>Spongiosa</td>
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<tr>
<td>Ventricularis/Artialis</td>
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</tbody>
</table>

Values are mean values ± SE of total leaflet thickness and individual leaflet layer thicknesses (in μm): fibrosa thickness, spongiosa thickness, and ventricularis (pulmonary only) or artialis (tricuspid only) thickness, for animals in the non-pregnant (NP), and pregnant (P) groups. Statistical comparisons were made between NP and P groups using t-Test comparisons of groups and p value ranges are indicated as follows: * = p < 0.05; ** = p < 0.01; *** = p < 0.001. n values for each parameter in the NP and P groups are presented in brackets for each valve.

**Collagen Crimp**

The belly region of the leaflet was analyzed using picrosirius-red stained sections. Remarkable changes were found in collagen fiber crimp with pregnancy (Table 4). Crimp length increased by 42% in the pulmonary valve, and 61% in the tricuspid valve during pregnancy. Similar crimp length increases were reported during pregnancy in the left-sided valves (39). There was a corresponding decrease in the percentage of the leaflet which was crimped in the pulmonary valve (also observed in the left-sided valves, (39))—crimped area decreased 28% in the pulmonary valve with pregnancy—but with no significant change in the tricuspid valve of pregnant animals.

<table>
<thead>
<tr>
<th>Table 4. Changes in collagen crimp with pregnancy</th>
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<tbody>
<tr>
<td>Collagen crimp: Wavelength, μm; Crimped Area, %</td>
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<tr>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Pulmonary Valve</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Non-pregnant</td>
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<tr>
<td>Crimp Wavelength</td>
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<td>% Crimped Area</td>
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</table>

Values are mean values ± SE of crimp wavelength (in μm) and percentage of leaflet area which is crimped (% Crimped Area) for animals in the non-pregnant (NP), and pregnant (P) groups. Statistical comparisons were made between NP and P groups using t-Test comparisons of groups and p value ranges are indicated as follows: * = p < 0.05; ** = p < 0.01; *** = p < 0.001. n values for each parameter in the NP and P groups are presented in brackets for each valve.

**DTT/HIT Experiments**
Collagen thermal stability was reduced in all heart valve tissues during pregnancy (Table 5). Specifically, $T_d$ decreased during pregnancy by 1.1°C and 1.9°C, in the pulmonary and tricuspid valves respectively. These reductions in denaturation temperature are similar to the decreases observed in the aortic (1.9°C) and mitral (2.3°C) valve leaflets (39), reflecting a decrease in collagen thermal stability across all four heart valves.

$T_d$ – Effect of Treatment. A two-way ANOVA demonstrated that collagen thermal stability, as indicated by $T_d$, was not significantly altered by NaBH$_4$ reduction in any pregnancy groups (data not shown). This indicates an absence of reducible, immature aldimine-derived crosslinks.

$t_{1/2}$ – Effect of Pregnancy. The half-time of load decay of HIT control specimens ($t_{1/2}$ control) increased by 126% in the pulmonary valve, and by 96% in the tricuspid valve during pregnancy (Table 5). This is in agreement with our previous observations on the left-sided valves: $t_{1/2}$ control increased in the aortic and mitral valve leaflets by 131% and 58% respectively (39). Together, these observations indicate an increase in mature crosslink content with pregnancy in all valves.

$t_{1/2}$ – Effect of Treatment in Pregnancy. In the NaBH$_4$-treated samples, pregnancy also resulted in a significant increase in load relaxation time in the pulmonary valve, but not the tricuspid. $t_{1/2}$ treated in the pulmonary valve increased by 77% during pregnancy—an increase comparable to that observed in the aortic (85%) and mitral (49%) valves (39). Together, these data indicate an increase in total collagen crosslinking (mature plus immature) during pregnancy in all valves except the tricuspid.

Another indicator of the relative maturity of the crosslinking present in a given tissue is the immature crosslink index: the ratio of $t_{1/2}$ treated (total crosslinking) to $t_{1/2}$ control (mature crosslinking). The higher this parameter, the greater the content of immature crosslinks. In non-pregnant animals, the immature crosslinking index was found to be small in the right-sided valves (2.3-2.6), in agreement with observations on the left-sided valves (39), suggesting a low content of immature crosslinks (Table 5). Surprisingly, this ratio was unchanged during pregnancy in the pulmonary valve, just as observed in the left sided valves. It was also surprising that there was a significant decrease in the immature crosslinking
index of the tricuspid leaflet during pregnancy. Indeed, the ratio approaches a value of 1.0 in pregnant animals suggests a nearly complete absence of immature crosslinks in the tricuspid leaflet (Table 5).

Leaflet Biochemical Composition

Effect of Pregnancy. Biochemical analysis of valve tissues demonstrated a significant alteration in leaflet composition in pregnancy, across all four valves (Table 6). Total collagen content, as a fraction of dry weight determined by hydroxyproline assay, increased during pregnancy for all valves. The percentage total collagen content increased by 8% in the aortic valve, 16% in the mitral valve, 11% in the pulmonary valve, and 26% in the tricuspid valve. The quantity of extractable collagen, investigated via the Sircol Bicolor Assay, was unchanged with pregnancy (data not shown).

<table>
<thead>
<tr>
<th>Table 6. Biochemical measures by percentage</th>
<th>Extracellular Matrix Content, % dry wt.</th>
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<tbody>
<tr>
<td>Aortic Valve</td>
<td>Mitral Valve</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>Non-pregnant</td>
</tr>
<tr>
<td>Pregnant</td>
<td>Pregnant</td>
</tr>
<tr>
<td>Total collagen</td>
<td>56.7 ± 3.0(9)</td>
</tr>
<tr>
<td>Elastin</td>
<td>6.2 ± 0.6(8)</td>
</tr>
<tr>
<td>sGAG</td>
<td>3.3 ± 0.5(4)</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>66.0 ± 3.3(9)</td>
</tr>
<tr>
<td>Pregnant</td>
<td>2.8 ± 0.4(8)</td>
</tr>
<tr>
<td>Pulmonary Valve</td>
<td>13.8 ± 0.7(5)</td>
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<tr>
<td>Non-pregnant</td>
<td>5.6 ± 0.6(8)</td>
</tr>
<tr>
<td>Pregnant</td>
<td>1.6 ± 0.2(8)</td>
</tr>
<tr>
<td>Tricuspid Valve</td>
<td>43.0 ± 2.2(10)</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>54.2 ± 1.9(14)</td>
</tr>
<tr>
<td>Pregnant</td>
<td>2.3 ± 0.2(6)</td>
</tr>
<tr>
<td>Elastin</td>
<td>0.8 ± 0.2(9)</td>
</tr>
<tr>
<td>sGAG</td>
<td>1.3 ± 0.2(4)</td>
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</table>

Values are mean values ± SE of biochemical extracellular matrix measures for animals in the non-pregnant (NP), and pregnant (P) groups: total collagen content (in %), sGAG content (in μg/mg tissue), and elastin content (in μg/mg tissue). Statistical comparisons were made between NP and P groups using t-Test comparisons of groups and p value ranges are indicated as follows: † = p < 0.1; * = p < 0.05; ** = p < 0.01; *** = p < 0.001. n values for each parameter in the NP and P groups are presented in brackets for each valve. *Results of total collagen content analysis for aortic and mitral valve leaflets previously presented by Pierlot et al. [39].

Elastin content, again expressed as per cent dry weight, decreased significantly in all valves during pregnancy: by 59% in the aortic valve, 55% in the mitral valve, 59% in the pulmonary valve, and 56% in the tricuspid valve. Similarly, there was a loss of sulfated GAG content with pregnancy across all valves: by 39% in the aortic valve, 52% in the mitral valve, 65% in the pulmonary valve, and 46% in the tricuspid valve. Water content was unchanged in pregnancy across all 4 valves (data not shown).

Effect of Valve Type. Examination of biochemical measures by heart side showed that total collagen content was 15% higher, sGAG content 98% higher, extractable collagen 57% lower, and elastin content 37% lower in left side heart valve leaflets compared to right side valve leaflets. On the other hand, total collagen content was 10% higher in outflow valve leaflets compared to inflow valves. All of these effects were conserved in pregnancy.

Cell Density and Phenotype

Effects of Pregnancy. Immunohistochemical studies showed a reduction in total cell (VIC) density in pregnancy (by approximately 24-29%) (Table 7). Surprisingly, though, the percentage of activated cells (i.e. those cells staining positive for alpha-smooth muscle actin) remained unchanged (Table 7). While the percentage of activated VICS—on average—did not change with pregnancy in any valve, in the mitral valve we observed a decrease in their density during gestation.
Effect of Valve Type. No significant differences in total cell density were found between left- versus right-side valves; however, comparison with our previous work showed greater density (43%) and proportion (61%) of activated cells in left side heart valves, and this difference was maintained in pregnancy. Thus, the higher-loaded, left-side valves possessed more “activated” VICs in both non-pregnant and pregnant animals. Looking at the data another way, outflow valves contained significantly higher densities cells, both: total cells (17%) and α-SMA+ cells (43%). These differences were again conserved in pregnancy.

<table>
<thead>
<tr>
<th>Table 7. Cell density and phenotype</th>
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<tr>
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<tr>
<td>Aortic Valve</td>
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<tr>
<td>Mitral Valve</td>
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<tr>
<td>Pulmonary Valve</td>
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<tr>
<td>Tricuspid Valve</td>
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<tr>
<td>Total Cell Density</td>
</tr>
<tr>
<td>Non-pregnant: 16.3 ± 1.8(8)</td>
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<tr>
<td>Pregnant: 11.5 ± 0.8* (15)</td>
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<tr>
<td>α-SMA+ Cell</td>
</tr>
<tr>
<td>Non-pregnant: 8.7 ± 1.7(8)</td>
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<tr>
<td>Pregnant: 5.6 ± 0.8 (15)</td>
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<tr>
<td>% α-SMA+ Cells</td>
</tr>
<tr>
<td>Non-pregnant: 52.6 ± 7.9(8)</td>
</tr>
<tr>
<td>Pregnant: 46.4 ± 3.8 (15)</td>
</tr>
</tbody>
</table>

Values are mean values ± SE of total cell number per 0.01 mm² (1 × 10⁴ cells mm²), α-SMA+ cell number per 0.01 mm² (1 × 10³ cells mm²), and percentage of α-SMA+ cells (% α-SMA+) for animals in the non-pregnant (NP), and pregnant (P) groups. Statistical comparisons were made between NP and P groups using t-Test comparisons of groups and p-value ranges are indicated as follows: * = p < 0.05; ** = p < 0.01; *** = p < 0.001; n values for each parameter in the NP and P groups are presented in brackets for each valve.

DISCUSSION

We have previously shown that the left-side valves of the maternal heart remodel during pregnancy. The valve leaflets increase in size, and show interesting changes in both composition and histological structure (38, 39, 60). In the present study we have extended our measurements to the right-side pulmonary and tricuspid valves, enabling: (i) left-side/right-side comparisons (ii) comparison by inflow/outflow valve position in the ventricles, and of course (iii) evaluation of the overarching effects of pregnancy on structuro-mechanical features. Beyond the right-side extension, we have added new assessments of matrix composition and cellularity in all four valves.

We can now state that the maternal heart valves largely remodel in similar fashion during pregnancy: with prodigious changes in leaflet dimensions, structure, ECM composition, and cellular density. Leaflets from all valves expanded in pregnancy (Figures 4, 5a), at least in part via increased production of collagen (Figure 5g and associated changes in the structure of the collagen network, i.e. loss of crimp (Figure 5c) and increased crimp length (Figure 5d, decreased denaturation temperature oddly coupled with valve-specific increases in crosslink maturity. Valve leaflets also thickened (Figure 5b), mainly in the fibrosa of the aortic, mitral, and pulmonary valves (Figure 5c). In early pregnancy, leaflet areal stretch declined for all valves; however (with the exception of the tricuspid valve), mechanical properties were regained by late pregnancy: a biphasic shift in extensibility. Matrix remodelling during pregnancy wasn’t limited to collagen. Both elastin and sGAG content decreased in all valves (Figure 5h,i). Curiously, while total cell...
density declined during pregnancy (Figure 5f), the proportion of those cells which showed the activated
cell phenotype (expressing α-SMA) was unchanged.

Since this is one of the very few comparative studies of all four valves in the mammalian heart, it is worth
noting that heart-side differences between valves were evident in many parameters assessed in this study.
For example, a two-way ANOVA demonstrated side-specific (aortic vs. pulmonary / mitral vs. tricuspid)
differences in leaflet area and thickness, extracellular matrix content, cellular phenotype, and collagen
thermal stability in non-pregnant animals. Much of this data speaks to the differences in the mechanical
loading environment between the systemic and pulmonary circulations in vivo (e.g peak transvalvular
pressure). Differences between inflow and outflow valves have also surfaced throughout. Inflow/outflow
differences (aortic vs. mitral / pulmonary vs. tricuspid) were seen in collagen content, crimp structure, cell
density, and mechanical properties. Here, the distinctions likely stem from the differing embryological
origins of these valve types (5, 12, 20, 22, 47).

Surprisingly, pregnancy-induced remodelling had virtually no effect on the relationships described above:
that is, between left- and right-side valves, or between inflow and outflow valves. Put another way, the
structuro-mechanical distinctions between the valves were conserved after pregnancy-induced remodelling.
Only one such relationship was altered in pregnancy. In non-pregnant animals, circumferential leaflet
dimensions were the same between left-side and right-side valves. In pregnancy, though, circumferential
length was significantly higher by 13% (left-side valves over the right-side valves)—even though all valve
leaflets enlarged. This result means that, on the higher pressure left-side of the heart, valve leaflets enlarge
via circumferential expansion to a larger degree than do the right-side valves.

Sidedness therefore seems to have limited influence on the potential for physiological remodelling of
valve leaflets—at least during pregnancy. This is a bit surprising. In previous in vitro studies with ovine or
porcine valves, Merryman et al. found that VICs isolated from left-side valves contained significantly
higher levels of α-SMA (cytoskeletal protein) and HSP47 (collagen synthetic protein) (32) and were
significantly stiffer (31) than were right-side valves. These results suggest that the cellular population of
left-side valves may be primed for enhanced remodelling in response to changes in tissue stress. As well,
Aldous et al. looked at bovine leaflet collagen from the four valves and reported that the collagen
denaturation temperature was inversely related to the transvalvular pressure gradient experienced in vivo
(2, 3). This result implies that valves exposed to higher pressures (i.e. left-side valves) may routinely
remodel faster than those that experience lower pressures (i.e. right-side valves) (3). In keeping with these
studies, our data have confirmed both the presence of a higher proportion of aVICs (expressing α-SMA)
and lower collagen denaturation temperatures in left-side valves. However, when challenged with the physiological changes associated with pregnancy, remodelling is nearly identical regardless of sidedness.

Our overall statistics have, when restricted to questions of sidedness or inflow/outflow position, described similar pregnancy-induced remodelling across valve types. These results stand in contrast with the clinical observation that about three times more women experience right-side regurgitation than experience left-side regurgitation (9). Returning to the full ANOVA results, we can isolate some intriguing, valve-specific remodelling effects associated with pregnancy. A good example is the remodelling of aortic valve leaflet area. Although all valves expanded, aortic valve expansion was strikingly greater (84%) than for any of the other valves (35-56%). It is possible that the enhanced areal remodelling response of the aortic valve is responsible for the lack of regurgitation with that valve during pregnancy, even with significant orifice dilation. A lesser expansive response in the mitral valve might still be sufficient for minimal regurgitation across that valve during pregnancy—if there were a sufficiently large reserve of redundant tissue (15, 60) that allowed the valve to maintain coaptation as the orifice expanded. Coaptation areas have only been reported in the literature for the aortic and mitral valves. Sohmer et al. reported a total coaptation area in the human aortic valve of 161 mm$^2$, with an average coaptation area for each of the three leaflets (trileaflet valve) of 54 mm$^2$, as measured by 3D echocardiography (49). In a similar study of the human mitral valve, Saito et al. (44) showed a total coaptation area of 160 mm$^2$, almost identical to that of the aortic valve. However, in that study, coaptation area was calculated by subtracting the closed leaflet area (mid-systole) from the total leaflet area (at the onset of valve closure), without consideration of the differential contributions of the anterior and posterior leaflets. In fact, the anterior mitral valve leaflet may on its own be sufficient to overlay the full valve orifice (37) and a greater coaptation reserve may be present for this valve, quite apart from the contribution of the chordae tendineae. It is possible that such a coaptation reserve is not present in the pulmonary and tricuspid valves. The reduced areal remodelling response which we observed in these valves would then result in the observed clinical regurgitation on the right side.

As another example, the tricuspid valve emerged as something of a special case. With the aortic and mitral valves, we had previously seen a loss of mechanical extensibility in early pregnancy but subsequent recovery in late pregnancy (39). We have confirmed this biphasic change in the pulmonary valve; however, in the tricuspid valve, areal stretch decreased in early pregnancy but was not regained by full term. Furthermore, in the tricuspid valve, we did not observe the same thickening of the fibrosa, decrease in percentage of leaflet area occupied by crimp, or increase in total crosslinking, that were found in all other valve types. Clearly the tricuspid valve remodels in a unique manner in pregnancy, although it is unclear why this should be the case.
Heart valve expansion has been previously reported mainly in cases of pathology or surgical intervention (e.g. pulmonary autograft after Ross Procedure (50), but little attention has focused on non-pathological alterations in leaflet size. The increases in leaflet area reported in the current study (as much as 84%) are much higher than the increases in mitral valve systolic leaflet area observed in patients with LV dysfunction (35%) (10), mitral regurgitation (resulting in chronic valve tethering) (30%) (44), or mitral valve regurgitation caused by experimental papillary muscle tethering in sheep (17% over 61 days) (11). Furthermore, when gestational timeline was considered in the current study, large increases in leaflet area occurred in early pregnancy (0-169d) in all valves except the pulmonary valve (which caught up in late pregnancy). Thus, leaflet expansion is overall rapid in pregnancy—an indication that other factors, beyond elevated stress, are driving this remodelling process. It is therefore likely that hormones play a significant role in modulating valve remodelling in pregnancy, particularly in early gestation.

The changes in leaflet size during pregnancy are, of course, only part of the story via which regurgitation is inhibited. Coaptation is achieved by leaflets of a given size which, when the valve is closed, stretch under the biaxial stress produced by transvalvular pressure. The approach taken in this and our previous studies has thus been to assess both dimensional changes and pregnancy-induced alterations in biaxial biomechanics. Biaxial mechanical data revealed a biphasic change in leaflet areal stretch: a leftward shift of the tension-areal stretch curve in early pregnancy, and a return to pre-pregnant values by late pregnancy. This occurred in the aortic, mitral, and pulmonary valves (although, as noted above, return was not complete in the tricuspid valve). Changes in circumferential extensibility were most important here. We speculate that the loss in circumferential extensibility in early pregnancy is due in part to the straightening (uncrimping) of the collagen network which causing leaflet lengthening, but at the cost of increased resistance to further extension. (See below.) It’s interesting that pulmonary leaflet areal stretch increased linearly with increasing gestational age. Thus, extensibility of the valve leaflet is regained during gestation via gradual structural remodelling of the leaflet tissue.

We have approached studies of structural remodelling by using quantitative histology, analysis of collagen thermal stability, and biochemical assays for matrix components. Cross-comparison of results from these various methods reveals some interesting insights into the remodelling process. Birefringence microscopy showed drastic increases in crimp length in all valves during pregnancy, with a synchronous overall loss in crimp structure in the collagen network. In the mitral valve in particular, there was close to a 3-fold increase in crimp length in pregnancy, with crimp structure becoming rarer (only 23% leaflet area remaining occupied by crimp) in the pregnant group. Thus, early increases in extensibility may be a creep-type phenomenon in existing leaflet collagen which is followed up by remodelling with newly-synthesized collagen. During valve remodelling, total collagen concentration (% dry weight, assessed by
hydroxyproline assay) increased by 8–26% across all valves. Thus, while the valve leaflets greatly
expanded in pregnancy, the quantity of collagen in the leaflet tissues not only kept up but actually
increased—and the collagen became more crosslinked. It is not surprising that as collagen was synthesized
in pregnancy, the fibrosa layer thickened (except, curiously, in the tricuspid valve where collagen content
increased but fibrosal thickness did not). By contrast, elastin and sGAG contents (again % dry weight)
decreased by 55–60% and 40–60% respectively in pregnancy. The magnitude of these changes is in rough
inverse proportion with the expansion in leaflet area. It is probable, therefore, that the actual quantity of
these molecules did not change dramatically, but that leaflet expansion and collagen accumulation
produced the observed decrease in concentration of elastin and sGAGs. Based on changes in leaflet size
and thickness, we estimate that leaflet elastin content actually decreased by 13–34% in pregnancy,
suggesting degradation does occur. In future, identification of the presence, as well as the up- or down–
regulation of the matrix metalloproteinases (MMPs) responsible for degradation of collagen and/or elastin,
as well as their tissue inhibitory metalloproteinases (TIMPs) would be beneficial in understanding valve
matrix turnover in pregnancy. A similar calculation indicates that sGAG content decreased by 40–60% in
pregnancy; however, it may have increased in the aortic valve only. GAGs are long-chain linear
polysaccharides that are negatively charged due to the presence of sulphate and uronic acid groups (34),
and are important in both water retention and organization of collagen fibre structure. In the current study,
water content was maintained during pregnancy, even though sGAG content decreased. It is possible that
non-sulfated hyaluronan content was preferentially maintained, allowing water content to be maintained.
This hypothesis requires further investigation. The preferential retention of sGAG in the aortic valve,
which experienced the greatest increase in leaflet area during pregnancy, suggests a role in remodelling of
the underpinning collagen network. Our data cannot specify the mechanism that may be at work here.

Increasing experimental evidence over the last decade has brought to light the importance of valvular
interstitial cells (VICs) and their diverse set of roles in physiological development, maintenance,
remodelling, and pathology. VICs are a dynamic and heterogeneous population of cells exhibiting
phenotypes of smooth muscle cells (SMCs), fibroblasts, and myofibroblasts (7, 41, 55) where
myofibroblasts are cells that display properties of both fibroblasts and SMCs (32, 33). In healthy adults,
fibroblast-like cells are the predominant cell type in valve tissues, with the few or no myofibroblasts (32,
46). Under altered physiological conditions, VICs can experience a phenotypic shift from quiescent
fibroblast to activated myofibroblast (or aVICs), distinguishable by high expression of contractile protein
markers not normally found in the interstitial cells of healthy valves: particularly α-SMA (7, 30). Elevated
aVIC expression (indicated by increased α-SMA) has been correlated to increased ECM turnover
(secretion and degradation) (53) and expression of both MMPs and (46, 53). Thus, we expected that a rise
in the proportion of aVICs in pregnancy would provide both a mechanism for, and indicator of, leaflet
remodelling. Remarkably, despite all the previous mentioned remodelling of heart valves in pregnancy,
leaflets from all four valves became less cellular during pregnancy. Total cell density decreased by 24–
29% across the valves. Further, there was no increase in the proportion of aVICs (α-SMA-positive cells).
These results were entirely unexpected. Similar to the reduction in elastin and sGAG content, absolute cell
numbers may have been maintained while the apparent loss of cell density was related to leaflet volume
expansion. We do not have paired leaflet size and cell count data to validate this hypothesis, although
averaged data across our discrete samples suggest that it may be valid. We had also expected to see a
significant shift in cell phenotype of the valvular interstitial cells (VICs) toward myofibroblast function
during remodelling. Again, this was not the case. Interestingly, though, while Schoen et al. reported that
only 2–5% of cells express α-SMA in normal human adult valves (1, 41, 46), the present data showed α-
SMA expression was very much higher in normal cattle: 52.6 %, 43.5 %, 29.4 %, and 27.5 % in the aortic,
mitral, pulmonary, and tricuspid valves respectively. It is not clear how this discrepancy arises, whether
due to species, sex, or differences in methodology. However, there may already be plenty of aVICs
present in valve tissue to accommodate the remodelling response in pregnancy.

While cellular phenotype is an indicator of the remodelling status in valve tissue, the hydrothermal
stability of tissue collagen can also be used as an indicator of collagen turnover rates. Since collagen is
crosslinked rather slowly after it is synthesized and assembled, and many (but not all) new crosslinks are
hydrothermally unstable, newly-assembled collagen is expected to be both less crosslinked and to possess
more immature, hydrolytically unstable crosslinks. We have examined this feature of valvular collagen
using hydrothermal isometric tension (HIT) testing which analyzes for biomechanically functional
crosslinking. When combined with NaBH₄ reduction, HIT testing can semi-quantitatively discriminate for
immature (hydrolytically unstable) crosslinks. Again, the results from hydrothermal testing were
surprising. The collagen denaturation temperature, Td, fell by 1.1–2.3°C during pregnancy. While this
reduction may seem small in absolute terms, it is very similar to the 1.3°C fall in Td seen in collagen of the
maternal pericardium during pregnancy (13), and similar in size to the rise in Td seen during perinatal
development of pericardium (36) or heart valves (2). A fall in denaturation temperature may indicate
reduced collagen crosslinking, but it can also indicate a destabilization associated with poor fibrillar
packing (reduced polymer-in-a-box stabilization (35)), or even reduced stabilization via other matrix
molecules like GAGs or proteoglycans (48). Further studies on the pregnancy-related changes in these and
other non-fibrillar collagenous components are warranted.

The isothermal relaxation phase of HIT testing provided a different window into crosslinking since its t₁/₂
parameter is a measure of total hydrothermally stable crosslinking present under 90°C water temperature.
These crosslinks may be physiologically mature or reduced to stability via NaBH₄. This technique produced one of the most surprising findings in the present study: an increase in total crosslinking of valvular collagen during pregnancy, combined with unchanged levels of immature crosslinking. (The exception again was the peculiar tricuspid valve where total crosslinking was unchanged in pregnancy, but the crosslinks were more hydrothermally stable.) These results are not consistent with significant turnover of collagen under normal physiological crosslinking mechanisms. As hypothesized in our study of the mitral valve, though, it is conceivable that valve collagen is crosslinked especially rapidly (38), but this notion stands to be proven. An additional possibility, discussed in depth previously (60), is the possibility of preferential deposition of the immature keto-amine crosslink HLKNL, which is hydrothermally (and acid) stable and therefore not classified properly by the HIT technique (4, 6). Aldous et al., who used both HIT and biochemical separation of crosslinks, reported that valve tissue from bovine steers contained significantly more keto-amine-derived crosslinks than aldimine-derived crosslinks (3). A similar combined analysis would greatly clarify this issue for valve remodelling in pregnancy. Whatever the biochemical basis for crosslinking profile during pregnancy, it is clear that collagen crosslinking is not decreased, say as a means to enable creep extension of the leaflets to compensate for orifice expansion.

Pregnancy produces volume overloading of the maternal heart: a feature also found in heart failure and other LV dysfunctions. However, based on the work here and in our previous studies, it is clear that pathways of remodelling (physiological versus pathophysiological) of the conditions are very different. In LV dysfunction, leaflet expansion occurs mainly through circumferential expansion. In pregnancy, by contrast, increased leaflet area occurs via increased extensibility along both axes of the leaflet: 19-41% increase circumferentially versus 11–30% radially. (10). Valve remodelling in heart failure produced reduced leaflet extensibility and is associated with increased concentrations of GAGs, cells, and DNA, decreased water content and viscosity, and increased amounts of more disorganized collagen, (16-18). Pregnancy is associated with increased collagen content, decreased crimp of collagen fibers, and a loss in extensibility; however the mechanical extensibility of the leaflet is regained in late pregnancy: an adaptation that never occurs in heart failure. Furthermore, pregnancy results in decreased concentrations of GAGs and cells, and maintained water content. It is clear that pregnancy and pathological heart disease follow distinctly different pathways of remodelling. Even if volume loading is a feature of both conditions, hormone profiles and signaling pathways of these conditions are distinct and produce distinctive responses.

In conclusion, by examining the pulmonary and tricuspid valves and by enhancing our analytic methods, we have now developed a unique dataset which describes dimensional, biomechanical, structural, and biochemical features of all four heart valves during pregnancy-induced remodelling of the bovine maternal heart. All four valves undergo significant adaptive remodelling, resulting in increased leaflet size, a
gestation-dependent biphasic shift in mechanical properties, an overall loss of collagen crimp, altered extracellular matrix composition and crosslinking profile, and decreased thermal stability—yet with no change in the proportion of cells expressing markers of the activated phenotype. For the most part, the remodelling response of all four valves was similar in pregnancy, with only limited influence of heart side or inflow/outflow position, and some interesting valve-specific effects. Importantly, this is the first study to show enhanced remodelling via leaflet expansion in the aortic valve, which may be responsible for resistance to regurgitation observed for this valve in clinical human pregnancy. The data has suggested a number of hypotheses which require further investigation. Certainly, identifying the triggers and mechanisms of physiological remodelling during pregnancy are fundamental to understanding valve pathologies, and to improving maternal health during pregnancy—and later in life.

CONFLICTS OF INTEREST: None

ACKNOWLEDGEMENTS

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FIGURE LEGEND

Figure 1. (a) Histological sectioning: each leaflet was dissected along the dotted lines to divide the leaflet in half and expose the belly region for sectioning. 5 μm serial sections were cut starting at the dotted lines to obtain circumferential sections for picrosirius red (PR) staining to examine collagen alignment and crimp, and radial sections for Verhoeff-Van Gieson (VVG) staining to identify leaflet layering and layer thicknesses, and immunohistochemical (IHC) staining of activated phenotype marker alpha-smooth muscle actin (α-SMA). Ruler scale is in cm. (b) Cell density and phenotype measurements were performed using two counter types: Type 1 counts all nuclei (total cell number); Type 2 counts α-SMA-positive nuclei (α-SMA+ cell number). Cells staining purple/blue are α-SMA-negative (no expression of α-SMA), while cells staining brown are α-SMA-positive (expression of α-SMA). Scale bar = 500μm.

Figure 2. Peak areal stretch at 60-N/m equibiaxial tension (net extensibility) of the pulmonary leaflet, plotted as a function of fetal crown-to-rump length (100 cm ~ term) along with measurements from NP heifers (open circles). Pregnant groups are shaded based on gestation: EP (0-169 days of gestation; shaded circles), MP (170-192 days; ½ shaded circles), and LP (193-270 days of gestation; dark circles). Significant regression line with 95% confidence intervals is shown for the fetal data.

Figure 3. Mean tension versus areal stretch curves for the (a) pulmonary, and (b) tricuspid valve leaflets, at each pregnancy state (NP, EP, and LP). Areal stretch is plotted as means ± SE at tension increments of 1.0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, and 60.0 N/m. Statistics shown with each pregnancy group are valid for every tension level above 5 N/m. a,bValues labeled with the same letter were not significantly different. n values are shown in Table 1 for areal stretch.

Figure 4. Representative images of leaflets of each valve, showing morphological changes with pregnancy. A, C, E, and G: leaflets from the non-pregnant (NP) group of the aortic, mitral, pulmonary, and tricuspid valves respectively. B, D, F, and H: leaflets from the pregnant (P) group of the aortic, mitral, pulmonary, and tricuspid valves respectively. Scale bar = 1 cm.

Figure 5. Summary of structural and mechanical parameters from all 4 valves in: NP heifers (open bars) and pregnant cows (filled bars) for aortic (AV), mitral (MV), pulmonary (PV), and tricuspid (TV) valves. A: leaflet area, B: total leaflet thickness, C: fibrosa thickness, D: Collagen crimp.
length, E: % crimped area, F: cell density, G: collagen content, H: Elastin content, I: sGAG content. Data for aortic and mitral valves in panels A-E, G from previous studies (39, 60). Data in panels F, H, I from Tables 7 and 8.
p=0.0001, r^2=0.6852