Physiological remodeling of the mitral valve during pregnancy

Sarah M. Wells,1,2 Caitlin M. Pierlot,1 and Andrew D. Moeller1

1School of Biomedical Engineering, Dalhousie University, Halifax, Nova Scotia, Canada; and 2Department of Physics and Atmospheric Science, Dalhousie University, Halifax, Nova Scotia, Canada

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Wells SM, Pierlot CM, Moeller AD. Physiological remodeling of the mitral valve during pregnancy. Am J Physiol Heart Circ Physiol 303: H878–H892, 2012. First published August 10, 2012; doi:10.1152/ajpheart.00845.2011. —There is growing evidence that heart valves are not passive structures but can remodel with left ventricular dysfunction. To determine if these tissues remodel under nonpathological conditions, we examined the mitral valve anterior leaflet during the volume loading and cardiac expansion of pregnancy using a bovine model. We measured leaflet dimensions, chordal attachments, and biaxial mechanical properties of leaflets collected from never-pregnant heifers and pregnant cows (pregnancy duration estimated from fetal length). Hydrothermal isometric tension (HIT) tests were performed to assess the denaturation temperature (Td) associated with collagen molecular stability and the load decay half-time (t1/2) associated with intermolecular cross-linking. Histological changes were examined using Verhoef–Van Gieson and picrosirius red staining with polarized light. We observed striking changes to the structure and material properties of the mitral anterior leaflet during pregnancy. Leaflet area was increased 33%, with a surprising increase (nearly 25%) in chordae tendineae attachments. There was a biphasic change in leaflet extensibility: it rapidly decreased by 30% and then reversed to prepregnant values by late pregnancy. The 2°C decrease in Td in pregnancy was indicative of collagen remodeling, whereas the 70% increase in HIT t1/2 indicated an increase in collagen cross-linking. Finally, histological results suggested transient increases in leaflet thickness and transient decreases in collagen crimp. This remodeling may compensate for the increased loading conditions associated with pregnancy by normalizing leaflet stress and maintaining coaptation. Understanding the mechanisms of mitral valve physiological remodeling in pregnancy could contribute to alternative treatments of pathological remodeling associated with left ventricular dysfunction.

heart valves; collagen; remodeling; cross-linking; pregnancy; mechanical properties

There is growing evidence that heart valves are not passive static structures but can instead adaptively remodel to alterations in their loading conditions. Evidence for heart valve remodeling has largely been associated with disease states such as LV dysfunction or heart failure. Much attention has been paid to the mitral valve, given the significance of mitral regurgitation in LV dysfunctions such as dilated cardiomyopathy and heart failure (9, 23, 33, 51). In these pathologies, altered cardiac structure and function, including annular dilation, are thought to elevate mechanical stresses on the mitral valve leaflets, which, in turn, induce “dysfunctional” remodeling of the tissue (17). In vivo sheep models of LV dysfunction reveal alterations in the gross structure and composition of the leaflets: leaflet area, thickness (9), and collagen synthesis (12) are increased, with decreases in collagen concentration, presumably due to greater increases in other extracellular matrix components (26). Similarly, explanted mitral valves from chronic heart failure patients are thicker and longer than those in control patients, with increased contents of collagen and cells and decreased water content (18). Correspondingly, these valves are stiffer, less extensible, and less viscous than normal valvular tissue (17). Together, human explant and in vivo sheep studies have demonstrated structural and mechanical alterations to the mitral valve that likely represent a compensatory response to pathological elevations in mechanical stress.

Less clear, however, is the remodeling capacity of mature heart valve tissues under nonpathological conditions. The purpose of the present study was to examine the remodeling capacity of mature heart valve leaflets in a nonpathological state, using the maternal circulation in pregnancy as a model. There are striking physiological cardiovascular changes in the maternal cardiovascular system of humans and other species as it accommodates the developing placenta and provides appropriate oxygen and nutrient delivery for the mother and fetus. In humans, blood volume is increased by ~40% during gestation, resulting in a volume overload state. The heart rate increases gradually over gestation by ~20–25%, whereas stroke volume rapidly increases by 30% over the first half of gestation and then plateaus (45). Cardiac output increases by ~50% by the third trimester, with more than half of that increase occurring in the first 8 wk of pregnancy (45).

Not surprisingly, the heart undergoes dramatic remodeling in early pregnancy (EP) as it adapts to an increased volume load. These changes resemble those associated with training and exercise, including LV hypertrophy (22, 45), with a 52% increase in LV mass (24) and increased dimensions of the atrial and ventricular chambers (11, 24, 45). The increase in cardiac dimensions includes annular dilatation of heart valves, with the orifice area increasing up to 14% in (human) aortic, pulmonary, and mitral valves (22, 44, 45). Dilatation of the heart valve annuli during pregnancy would be expected to trigger remodeling of the valve leaflets due to the associated increases in

HEART VALVES have an important physiological function in maintaining unidirectional blood flow and preventing regurgitation. The mitral valve in particular is closed during ventricular systole, preventing backflow of blood into the left atrium as the left ventricle (LV) ejects the stroke volume into the aortic outflow tract. Heart valves function under harsh mechanical demands, opening and closing ~35 million times in 1 yr and supporting high transvalvular pressures across the closed leaflets. These functions translate into a complex combination of compressive, shear, and tensile stresses within the valve leaflet tissues. Finite-element models have shown that tensile stresses caused by transvalvular pressure are the most dominant on the valve leaflets (26), with peak transvalvular pressure corresponding to maximal engagement and loading of its collagen fibers (48).

Address for reprint requests and other correspondence: S. M. Wells, School of Biomedical Engineering, Dalhousie Univ., 5981 University Ave., Halifax, NS, Canada B3H 4R2 (e-mail: sarah.wells@dal.ca).
tensile stresses. Indeed, finite-element models of the aortic and mitral valves have shown that orifice dilation decreases leaflet coaptation and increases their radius of curvature and tensile stress, via the law of Laplace (16, 27). An 18% annular dilatation, similar to that during human pregnancy, results in a more than twofold increase in stress in both the anterior and posterior leaflets (27).

It is interesting that, despite the large increase in valve orifice area, mitral regurgitation is relatively uncommon in pregnancy (7, 46). One possible explanation for this is that the mitral valve has a large “functional reserve,” with a total leaflet area that is at least 1.5 times larger than that of the annulus (15). This surplus area, located in the coaptation surface, allows the valve to experience orifice expansion under various hemodynamic conditions, such as volume loading, without developing mitral regurgitation (38). Another possibility is that the mitral leaflets undergo adaptive remodeling during pregnancy, increasing leaflet area to maintain coaptation.

The objectives of the present study were to determine changes in the structure and mechanical properties of the bovine mitral valve anterior leaflet during pregnancy. Bovine tissues are commonly used in studies on cardiovascular tissue mechanics, especially those on native and chemically modified heart valve and pericardial tissues. This model, however, is advantageous for several other reasons. First, the bovine cardiovascular system makes adaptations to pregnancy similar to those in humans and other mammalian species, including blood volume expansion (41) and hypertrophic remodeling of the heart (49). Second, bovine tissues obtained at slaughter are a consistent and reliable source of tissue from pregnant animals, especially where the age and reproductive history of the animal are obtainable.

Third, the duration of bovine pregnancy may be estimated from the well-established relationship between the crown-to-rump length of the fetal calf and its gestational age (13). Finally, the bovine model provides heart valve leaflet samples large enough for biaxial mechanical testing.

We hypothesized that, during pregnancy, the maternal bovine mitral valve undergoes adaptive remodeling similar to that observed in heart failure and other LV dysfunctions where chamber and orifice expansion take place. In particular, we hypothesized that the anterior leaflet enlarges during pregnancy with decreases in biaxial extensibility and that these changes will be associated with thermomechanical alterations to collagen that indicate structural remodeling and adaptation to elevated stress. Thus, we hypothesized that the mitral anterior leaflet collagen thermal stability will decrease, indicative of collagen turnover and remodeling, and collagen cross-linking will increase with the elevated leaflet stress during pregnancy. Here, we report the changes in 1) leaflet morphometry and quasistatic biaxial mechanical properties, 2) collagen thermal stability, and 3) collagen cross-linking of the mitral valve anterior leaflet in pregnant cows and nonpregnant (NP) heifers. Changes in these parameters were also assessed as a function of pregnancy duration.

**METHODS**

**Tissue Harvest and Sample Preparation**

All tissue harvest procedures were approved by the Animal Care Committee of Dalhousie University and were conducted in accordance with guidelines approved by the Canadian Council of Animal Care.

Bovine heart valve leaflets and valvular roots were harvested fresh from slaughter for food a local abattoir (Armstrong Food Services, Kingston, NS, Canada). Hearts were collected from heifers (cows that have reached sexual maturity and have never been pregnant, between the ages of 1 and 2 yr old) and from pregnant cows. Heart mass, volume, transverse circumference, and apical circumference were measured. From pregnant cows, fetal crown-to-rump lengths were measured, from which pregnancy duration was estimated for each cow (13). A full-term bovine fetus is ~100 cm in length. Cows were divided into three groups: NP, EP, and late pregnancy (LP) according to their reproductive status and pregnancy duration (using fetal crown-to-rump length).

The mitral valve anterior leaflet was chosen since it has been shown to experience larger stresses than the posterior leaflet (25). The leaflets were excised as close as possible to the valve root, washed in Hanks’ physiological solution, and placed in Falcon tubes for transport. Tissues were transported back to the laboratory in Hanks’ physiological solution including 6 mg/l trypsin inhibitor (lyophilisate) and an antibiotic-antimycotic agent containing 10,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B (all chemicals from Sigma-Aldrich Canada, Oakville, ON, Canada).

**Leaflet Morphometric Analysis**

Each excised anterior leaflet was laid flat on a corkboard with the ventricular side facing up (Fig. 1A). Stick pins were placed at the leaflet insertion site of each chordae tendinae to mark J) the primary “strut” chordae; 2) the secondary chordae, with diameters between 1 and 3 mm; and 3) the tertiary chordae, with diameters of <1 mm (Fig. 1B). The very smallest chordae (i.e., small side branches or tissue that was translucent) were not counted. Digital images of the leaflets, pins, and accompanying scale bar were captured with a Nikon SMZ 800 dissection microscope and a Nikon Coolpix 995 camera (Nikon, Tokyo, Japan). Digital images were then imported into image-analysis software (ImageJ, National Institutes of Health), and midline radial and circumferential lengths were measured. The freehand draw tool was used to trace the perimeter of the leaflet to obtain total leaflet area (Fig. 1A). The distance between the two primary strut chordae (“strut distance”) and the position of this midline from the leaflet attachment site (“strut position”) were also measured (Fig. 1B).

**Biaxial Mechanical Testing**

Leaflets were stored at 4°C in Hanks’ physiological solution with protease and bacterial inhibitors as described above for no longer than 72 h until they were used for biaxial testing. A square sample of tissue was excised from the center belly region of the anterior leaflet between the attachment sites of the two prominent strut chordae tendinae (Fig. 1A), in an attempt to ensure a sample of similar mechanical characteristics. To prepare the samples, ~3 mm was trimmed from the base and the free edge of the leaflet.

Biaxial testing was carried out on a custom-built, servo-hydraulic biaxial testing apparatus (MTS, Eden Prairie, MN), as previously described (53) except with the use of suture-style grips (53, 55). The device consists of two opposing pairs of servo-hydraulic actuators aligned orthogonally. Tests were carried out in biaxial configuration, using all four actuators. The two actuator pairs provided two axes, allowing independent deformation of the test samples in two perpendicular directions. Actuator waveforms were generated using T/RAC waveform generation (MTS). An actuator in each pair had an attached GSO series 1,000-g cantilever load cell (Transducer Techniques, Temecula, CA). The time, actuator position, and load data were acquired using a custom-written program under LabView software and a 12-bit analog-to-digital (A/D), digital-to-analog (D/A) PCI-6035E card (National Instruments, Austin, TX) on a Macintosh computer (Power Mac G4, Apple Computer, Cupertino, CA).
The circumferential direction of the leaflet. Scale bars indicate the mechanical testing and marker placement. Note the large collagen fiber bundles in the sample in the shape of a rectangle. A: image of an isolated mitral anterior leaflet showing the location of stick pins marking the insertion sites of strut chordae (S), secondary (2°) chordae (diameters > 1 mm), and tertiary (3°) chordae (diameters < 1 mm). The strut position was measured as the vertical distance between the midline connecting the two primary strut chordae and the attachment edge. B: image of an isolated mitral anterior leaflet showing dimensional measurements taken using ImagemJ. The measurements taken were leaflet area (dashed outline), circumferential length (l{circ}), and radial length (l{rad}). Also shown is the region excised for biaxial mechanical testing and marker placement. Note the large collagen fiber bundles in the circumferential direction of the leaflet. Scale bars = 1 cm.

Video images of the sample surface were recorded with a charge-coupled device camera (Cohu 4190, San Diego, CA) to subsequently track surface deformation markers using ImagemJ on a separate Macintosh computer (Power Mac G4, Apple Computer). Video capture was achieved at 15 Hz, and video and analog data were synchronized by a 5-V digital pulse originating from LabView via the D/A board.

A 000 silk suture with a 3-mm cutting needle was tied to one of five nylon pulleys of one grip. The suture was then inserted into four pins marking the insertion sites of strut chordae (S), secondary (2°) chordae (diameters > 1 mm), and tertiary (3°) chordae (diameters < 1 mm). The strut position was measured as the vertical distance between the midline connecting the two primary strut chordae and the attachment edge. B: image of an isolated mitral anterior leaflet showing dimensional measurements taken using ImagemJ. The measurements taken were leaflet area (dashed outline), circumferential length (l{circ}), and radial length (l{rad}). Also shown is the region excised for biaxial mechanical testing and marker placement. Note the large collagen fiber bundles in the circumferential direction of the leaflet. Scale bars = 1 cm.

The graphite marker positions on the surface of the tissue were analyzed using a custom-written program under ImagemJ. The pixel coordinates of the particle centers were used to determine the stretch ratios previously defined in (55) using a custom-written MathCAD program (Parametric Technology, Needham, MA) written by Dr. Michael Sacks (University of Pittsburgh). The biaxial extensibility was characterized by the axial stretch ratios [l{circ}^{\text{peak}} (circumferential) and l{rad}^{\text{peak}} (radial)] under peak equibiaxial membrane stress (60 N/m). The net extensibility of the tissue membrane was represented by the areal stretch under 60-N/m equibiaxial tension, which was calculated as follows:

\[
\text{Areal stretch} = \left( \frac{l^{\text{peak}} \times l^{\text{peak}}}{l^{\text{peak}} - l^{\text{peak}}} - 1 \right) \times 100\% \quad (1)
\]

The 60-N/m equibiaxial tension was chosen as it closely represents the deformation under peak diastolic load and it facilitates comparisons with a related study (55). To assess the quasistatic elastic properties of the valve tissue over the entire loading range examined, 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.

Denaturation Temperature Testing and Hydrothermal Isometric Tension Testing

Tissue preparation and sodium borohydride reduction. Denaturation temperature (T_d) tests (DTT) and hydrothermal isometric tension (HIT) experiments were conducted on the custom-built apparatus described by Lee et al. (31) following the modified protocol of Wells et al. (54) to determine T_d and proportions of immature to mature cross-links. After biaxial mechanical testing, the square tissue sample was bisected circumferentially, producing two pieces of tissue with equal dimensions. One piece served as a control sample, and the other was treated with sodium borohydride (NaBH_4) to reduce and stabilize immature, thermally labile cross-links (2, 43, 54). Comparison of thermomechanical behavior with and without NaBH_4 stabilization provided an assessment of the relative contribution of immature cross-links. The NaBH_4 stabilization procedure consisted of four 15-min treatments in a 0.1 mg/ml NaBH_4-borate buffer solution (pH 9.0) at 4°C with constant agitation. Untreated samples were subjected to the same conditions (pH, buffer, temperature, and agitation) except in the absence of NaBH_4. Untreated and stabilized samples were then rinsed in Hank’s solution for 15 min at 4°C with constant agitation before thermomechanical testing. Finally, samples were washed for 10 min in Hank’s physiological solution.

DTT/HIT testing. The DTT/HIT testing allowed for the simultaneous testing of up to six samples, as previously described (2, 54). Briefly, tissue strips were gripped between two spring clamps, where one clamp was attached to a rigid fixture and the other clamp was attached to an adjustable fixture attached to a strain gauge, cantilever load cell. Samples were extended to an initial load of 50 g and then held under isometric constraint for the duration of the test. Mounted samples were immersed in a 4-liter beaker of distilled water, and the bath temperature was monitored with a centrally located thermistor probe positioned at the level of the samples. The water was heated with a Cimarec 2 plate heater (Barnstead-Thermolyne, Dubuque, IA) from room temperature at a rate of ~1°C/min to a 90°C isotherm. The temperature was held at this isotherm, 90 ± 0.5°C, for 3 h by an on/off heater control. Time, temperature, and load data were acquired, and heater control was achieved, using a custom-written LabView pro-
gram on a Dimension 4800 computer (Dell Computing, Round Rock, TX) with a 12-bit A/D, D/A DAQ board (model NB-MIO-16L, National Instruments). Data points were collected at intervals of 1°C during the temperature ramp and at 30-s intervals during the isotherm portion of the test.

Analysis of DTT/HIT data. $t_{1/2}$ of each sample was determined as the temperature at the first data point marking the beginning of a consistent rise in load as temperature was increased from room temperature to 90°C (54).

Data recorded at 30-s intervals during the 3-h 90°C isotherm was used to calculate the half-time of the exponential load decay ($t_{1/2}$). Le Louët et al. (30) previously described this as a Maxwell-type relationship using the following equation:

$$L(t) = L_o e^{-kt}$$

where $L(t)$ is the load at time $t$, $L_o$ is the maximum load attained at the isotherm onset, and $k$ is a constant denoting the slope of the curve. The following equation was used to calculate $t_{1/2}$:

$$t_{1/2} = \frac{\ln 2}{k}$$

The logarithm of $L(t)/L_o$ was plotted against time (during the 90°C isotherm), and $k$ was obtained over an 8,000-s time interval between the 2,000- and 10,000-s mark in the isotherm. This data interval was chosen because it represented an extremely linear portion of the logarithmic load decay curve, avoiding any thermoelectric contraction, which sometimes occurred at the beginning of the isotherm. The $t_{1/2}$ parameter was calculated from $k$ using previously described methods (54).

Histological Analysis

Another set of mitral valve leaflets was collected from pregnant cows and NP heifers as described above. The anterior leaflets were divided in half along the radial direction, fixed from fresh in 10% neutral buffered formalin for a minimum of 48 h, embedded in paraffin, sectioned into 5-μm sections, and mounted on slides. One half of each leaflet was sectioned circumferentially for picrosirius red staining to examine collagen alignment and crimp, whereas the other half was sectioned radially for Verhoeff-van Gieson (VVG) staining to identify leaflet layering and elastin fibers (VVG Elastin Staining Kit, Polysciences, Washington, PA). Sections were deparaffinized, rehydrated, stained in Verhoeff’s solution for 1 h, and differentiated in 2% ferric chloride for 1 min followed by several water washes, a counterstain, and dehydration for slide mounting. For picrosirius red staining, sections were deparaffinized, rehydrated, and then stained for 1 h with 0.1% picrosirius red solution. After staining, slides were rinsed with several water washes and then dehydrated for mounting.

Images were taken using a Nikon Eclipse E600 light microscope equipped with a polarized light filter and a Nikon Coolpix 995 digital camera (Nikon).

Statistics

Results are expressed as means ± SE, and $n$ values used in the calculations are the numbers of animals providing samples for each group. Data collected from heifers (never-pregnant female cattle) were classified as NP. Data collected from pregnant cows were subdivided into two groups: EP and LP according to the fetal crown-to-rump length. Cows carrying fetuses with crown-to-rump lengths of 0–50 cm were classified as EP, and those carrying fetuses over 50 cm were classified as LP. By gestational age, these groups correspond to ~0–159 days of gestation for EP and 160–270 days of gestation for LP. To determine the differences between pregnancy groups, one-way ANOVA was performed followed by Tukey honestly significant difference comparisons among the three groups (the NP group and the two pregnancy groups). In the case of HIT data, this was preceded by two-way ANOVA that demonstrated no interactions between the factors of treatment (control vs. NaBH₄) and pregnancy state (NP, EP, and LP).

To assess the changes in any parameter as a function of pregnancy duration, data for each parameter were plotted as a function of gestational age and fitted with a least-squares linear regression. The regression was considered significant when $P < 0.05$. Statistical analyses were performed using JMP Statistical Software (version 5.0.1.2, SAS Institute, Cary, NC).

RESULTS

Cardiac Dimensions

Bovine hearts underwent significant increases in mass, volume, and dimensions by LP. Heart mass in NP heifers was 2.13 ± 0.16 kg and remained unchanged during EP. In LP animals, however, heart mass was 44% greater than that of NP animals (Table 1). Similarly, heart volume was unchanged from NP animals (2.24 ± 0.17 liter) in EP but was increased 107% in LP animals (3.31 ± 0.26 liters) from NP values. Enlargement of the heart in LP occurred in both the circumferential and base-to-apex directions. For NP animals, mean heart circumference was 45.9 ± 1.3 cm in the transverse direction and 53.0 ± 1.2 cm in the apical direction. These values were increased 15% in LP animals from those of NP animals (Table 1).

Leaflet Morphology

The anterior leaflet of the bovine mitral valve underwent rapid and striking morphological changes during pregnancy, with increases in leaflet area and number of attachments by chordae tendinae. Figure 2 shows representative images of anterior mitral leaflets from a NP heifer (A), a pregnant cow at 94 days of gestation (Fig. 2B), and a near-term (261 days of gestation) cow (C). Mean anterior leaflet area increased 33% from NP animals (11.1 ± 0.6 mm²) to EP animals (14.8 ± 1.0 mm²) and then remained unchanged in LP animals (Fig. 3A). Increases in leaflet area were caused by rapid leaflet expansion during EP, in both the radial and circumferential directions. Radial leaflet length increased 20% from NP animals (2.5 ± 0.1 mm²) to EP animals (3.0 ± 0.2 mm²; Fig. 3B), and

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<th>Table 1. Summary of changes in heart mass, volume, and dimensions with pregnancy</th>
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<td><strong>NP Group</strong></td>
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Values are mean values ± SE of cardiac mass, volume, and dimension parameters for animals in the nonpregnant (NP; $n = 11$), early pregnant (EP; $n = 9$), and late pregnant (LP; $n = 4$) groups. Statistical comparisons were made among NP, EP, and LP groups using ANOVA followed by Tukey’s honestly significant difference (HSD) multiple-comparison method. **Within each parameter, values labeled with the same letter were not significantly different.**

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Fig. 2. Representative images of the anterior mitral valve leaflet showing morphological changes with pregnancy and its duration. A: leaflet from the nonpregnant (NP) group (never-pregnant heifer). B: leaflet from a cow in the early pregnant (EP) group (94 days of gestation, ~1/3 through gestation). C: leaflet from a cow in the late pregnant (LP) group (261 days of gestation, close to full term). Scale bars = 1 cm.

circumferential length increased 14% from NP animals (5.1 ± 0.1 mm²) to EP animals (5.8 ± 0.3 mm²; Fig. 3C).

While increases in anterior leaflet area during pregnancy were prominent, changes in the position and separation of the primary strut chordae were less so, likely a result of the large inherent variability in their anatomy. ANOVA comparisons between NP, EP, and LP animals yielded \( P \) values of 0.19 for strut position and 0.06 for strut distance. However, increases in both parameters were revealed when comparisons were made between NP and (pooled) pregnant animals. The strut position in NP animals was 11.0 ± 0.6 mm (\( n = 9 \)) and was increased to 12.7 ± 0.6 mm (\( n = 12 \)) in pregnant animals (\( P = 0.037 \)). Similarly, there was a trend (\( P = 0.06 \)) toward increased strut distance in pregnant animals (27.8 ± 5.7 mm, \( n = 12 \)) compared with NP animals (22.8 ± 5.7 mm, \( n = 9 \)). However, when the strut position and distances were normalized to leaflet radial and circumferential lengths, respectively, they were unchanged with pregnancy, suggesting a uniform enlargement of the leaflet, at least between the primary struts and the attachment edge.

Accompanying the increase in leaflet area was a corresponding increase in the number of chordal tendinae to the ventricular surface. The total number of chordae attached increased linearly with pregnancy duration (fetal length; Fig. 3D), with mean values increasing by 22% from the NP state (18.3 ± 0.3) to the LP state (22.3 ± 1.3; Fig. 3E). This was a result of increases in the number of both secondary and tertiary chordae during pregnancy. The number of secondary chordae was significantly increased from NP animals (6.9 ± 0.4) to EP animals (8.0 ± 0.5), whereas the number of tertiary chordae was significantly increased from NP animals (8.9 ± 1.4) to LP animals (11.8 ± 2.9).

While both leaflet area and chordal attachments increased during pregnancy, the number of chordal attachments did not keep in step with the increase in leaflet area. There was a progressive decrease in the density of chordal attachments (number of attachments/cm²) during pregnancy, with mean values decreasing 27% from the NP group (1.5 ± 0.1 attachments/cm²) to the LP group (1.1 ± 0.1 attachments/cm², \( P = 0.049 \)).

Leaflet Mechanical Properties

In all mitral valves from NP and pregnant animals, anterior leaflets were more extensible along the radial axis than the circumferential axis (Fig. 4), as previously reported in other species (sheep and pigs; Table 2). The net extensibility (represented by peak areal stretch under 60-N/m equibiaxial tension) of anterior mitral valve leaflets significantly decreased during EP and returned to prepregnant values by term (Fig. 5, A and B; mean values and statistical comparisons are also shown in Table 2). There was a rapid decrease in peak areal stretch in early pregnancy, with mean values decreasing 30% from the NP group (2.30 ± 0.11) to the EP group (1.91 ± 0.14; Table 2 and Fig. 5B). Areal stretch then increased linearly with fetal development (\( P = 0.018 \); Fig. 5A), with mean values returning to prepregnancy values by LP (2.36 ± 0.10; Table 2 and Fig. 5B).

The reversible decrease in leaflet extensibility in EP was produced by changes in leaflet extensibility in both the circumferential and radial directions (Table 2 and Fig. 5, C–F). \( \lambda_{c}^{\text{peak}} \) under 60 N/m equibiaxial tension decreased significantly from NP values (1.37 ± 0.05) by EP (1.23 ± 0.05; Table 2 and Fig. 5D). \( \lambda_{c}^{\text{peak}} \), while not significantly altered from NP values (1.67 ± 0.06) by EP, rapidly and significantly increased over pregnancy duration (as assessed by fetal length, \( P = 0.036 \); Fig. 5E), with mean values increasing 14% by LP (1.75 ± 0.07; Table 2 and Fig. 5F).

In addition to observations at peak equibiaxial tension, the reversible decrease in anterior leaflet areal stretch in EP was also observed over almost the entire range of equibiaxial tension used in our experiments. Figure 6 shows mean areal stretch at each equibiaxial tension level for the NP, EP, and LP groups. At each equibiaxial tension level, above 5 N/m, mean areal stretch was decreased in EP animals from those of NP or LP animals (Fig. 6). The result was a significant shift to the left...
Collagen Cross-Linking (HIT Results)

**Effects of Pregnancy.** Collagen thermal stability decreased rapidly in EP. (Control) $T_d$ was significantly decreased from the NP group (68.6 ± 0.5°C) to the EP group (66.4 ± 0.6°C) and remained unchanged into the LP group (66.2 ± 0.4°C, $P < 0.001$).

**Effects of NaBH₄ Treatment.** NaBH₄ treatment did not alter collagen thermal stability in any of the pregnancy groups. Results from two-way ANOVA revealed no effect of NaBH₄ treatment on $T_d$ of the tissue in any of the pregnancy groups (data not shown).

**$t_{1/2}$.** Effects of Pregnancy. Load relaxation (in control tissues) during the 90°C isotherm became progressively slower from the NP state to the LP state, implying an increase in the content of mature collagen cross-links over this interval. There was a trend toward a significant linear increase in HIT $t_{1/2}$ control with pregnancy duration (Fig. 7A), resulting in a progressive 70% increase in mean HIT $t_{1/2}$ control values from the NP group (8.6 ± 1.1 h) to the LP group (15.0 ± 1.9 h, $P = 0.02$; Fig. 7B).

**Effects of NaBH₄ Treatment.** NaBH₄ treatment significantly increased HIT $t_{1/2}$ in all of the pregnancy groups ($P = 0.01$), as indicated by the solid lines connecting the control and NaBH₄ values within each pregnancy group in the interaction plot shown in Fig. 8. The increase in $t_{1/2}$ after NaBH₄ treatment reflects the presence of immature collagen cross-links in the mitral anterior leaflet at all pregnancy states. As in previous studies, the relative increase in $t_{1/2}$ after NaBH₄ treatment (i.e., the ratio of $t_{1/2}$ in NaBH₄ to $t_{1/2}$ in control) was used as an indicator of the ratio of immature to mature collagen cross-linking. This ratio was relatively low and, surprisingly, unchanged across pregnancy groups (mean values of $t_{1/2}$ in NaBH₄ divided by $t_{1/2}$ in control: 1.4 ± 0.2 in the NP group, 1.7 ± 0.3 in the EP group, and 1.6 ± 0.3 in the LP group, $P = 0.63$), suggesting that the level of immature collagen cross-linking remained at a relatively low and constant level during pregnancy.

**Effects of NaBH₄ Treatment × Pregnancy.** While the mitral valve tissue showed an increase in HIT $t_{1/2}$ in control by LP, there was no change in $t_{1/2}$ in NaBH₄-treated tissue between pregnancy states, suggesting that while mature cross-links increased during pregnancy, total cross-linking remained unchanged. Paradoxically, this implies that the amount of imma-
tured cross-linking changed very little (or if anything was decreased) from the NP state to the LP state. Results from two-way ANOVA showed that there was no interaction between the factors of NaBH4 and pregnancy state ($P = 0.46$; Fig. 8). The similar but modest increase in $t_{1/2}$ across pregnancy states suggests that the content of immature cross-linking was relatively low and, surprisingly, unchanged during pregnancy.

**Leaflet Histological Properties**

Histological analyses showed significant changes in the trilayered structure and overall thickness of the anterior mitral leaflet during pregnancy. Figure 9 shows representative VVG-stained radial sections taken from the belly region of the leaflet. Full leaflet thickness is shown for a NP heifer in Fig. 9A, with higher-magnification images of the atrialis, spongiosa, and ventricularis shown in Fig. 9, B–D. The atrialis (Fig. 9B) was characterized by a large number of densely stained elastic fibers (stained black) and associated dense collagen. The spongiosa (Fig. 9C) was composed of a network of fine elastic and collagen fibers with large pores, which contain mainly hydrated glycosaminoglycans and proteoglycans (not stained by VVG). The ventricularis (Fig. 9D) was composed mainly of collagen (stained pink-purple), with valvular interstitial cells (nuclei stained black) with a thin outer layer of elastic fibers that was highly variable in thickness and density.

In EP (113 days of gestation; Fig. 9, E–H), the leaflet was much thicker, largely through thickening of the dominant, densely collagenous ventricularis. The atrialis was less dense, with sparse staining of elastic fibers (Fig. 9F), whereas the extracellular matrix of spongiosa showed some densification, with increased elastin and collagen staining and an elongation of its pores in the radial direction (Fig. 9G). In contrast, in LP (184 days of gestation; Fig. 9, I–L), the leaflet was reduced in thickness from that in EP, with further densification and elongation of the spongiosa (Fig. 9K) and a slightly more dense atrialis. In the near-term animal (Fig. 9; M–P), elongation of the spongiosa (Fig. 9O) and densification of the atrialis (Fig. 9N) were even more prominent.

Observations from picrosirius red-stained sections also suggested striking changes during pregnancy in collagen fiber crisp in the circumferential direction. Tissue from NP animals revealed a uniform, crimped structure with a period of ~15 μm (Fig. 10, A and B). This structure was much less spatially uniform in EP, with little or no change in the crimp period (Fig. 10, C and D). At the beginning of the LP period, there was a dramatic loss of collagen crisp, as shown in the tissue from a near-term animal (184 days of gestation; Fig. 10, E and F). Collagen crisp was regained near term (Fig. 10, G and H), but at a period almost double that in the NP animal.

**Discussion**

This study reported, for the first time, dramatic, adaptive remodeling in mature heart valve leaflets under nonpathological conditions. The anterior leaflet of the bovine mitral valve undergoes rapid increases in leaflet size and chordal attachments that may accommodate not just the increased orifice area but the inevitable increases in leaflet and chordal stresses that would accompany this expansion. Accompanying this increase in size was a surprising, biphasic change in leaflet extensibility: decreasing in EP and then reversing back to prepregnant values by LP. The results from this study suggest that the volume loading and valve orifice expansion during pregnancy trigger

## Table 2. Summary of leaflet mechanical properties from the present and previous studies

<table>
<thead>
<tr>
<th>Reference(s)</th>
<th>Porcine Leaflets</th>
<th>Ovine Leaflets</th>
<th>Bovine Leaflets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biaxial</td>
<td>Flow loop</td>
<td>In situ (10)</td>
</tr>
<tr>
<td>$\lambda_c^{peak}$</td>
<td>32*</td>
<td>20, 48</td>
<td>10</td>
</tr>
<tr>
<td>$\lambda_{p, peak}$</td>
<td>1.12*</td>
<td>1.08–1.1</td>
<td>1.02–1.04</td>
</tr>
<tr>
<td>$\lambda_c^{peak}$</td>
<td>1.26*</td>
<td>1.30–1.32</td>
<td>1.14</td>
</tr>
<tr>
<td>$\lambda_c^{peak}$</td>
<td>0.87*</td>
<td>0.83</td>
<td>0.90</td>
</tr>
<tr>
<td>Areal stretch</td>
<td>N/A*</td>
<td>1.42–1.47</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Shown are mitral anterior leaflet mechanical properties from the present study and values obtained from previous studies for comparison. Peak circumferential stretch ratio ($\lambda_c^{peak}$), peak radial stretch ratio ($\lambda_{p, peak}$), the ratio of these values ($\lambda_c^{peak}/\lambda_{p, peak}$), and peak areal stretch are shown. Data from previous studies are shown for porcine leaflets tested in a physiological left ventricular simulating flow loop (20, 48), and in an isolated porcine heart preparation in situ (10). Data are also shown for ovine anterior leaflets assessed in vivo using the excited, stress-free leaflet as the reference state (3). Data from the present study are mean values ± SE for bovine NP, EP, and LP groups. For each parameter, statistical comparisons were made between pregnancy groups from the present study using ANOVA followed by Tukey HSD multiple-comparison method. a, bValues labeled with the same letter were not significantly different. *Significant difference from our mean NP value ($P < 0.05$). N/A, not applicable.
remodeling and tissue growth that increases the size and alters the material properties of the leaflet.

We observed a rapid 33% increase in leaflet area within the first 2 mo of bovine pregnancy. This is similar to the 35% increase in systolic leaflet area reported in an echocardiographic assessment of patients with LV dysfunction (9). Elevated leaflet stress (in pregnancy or LV dysfunction) is likely the trigger for physiological or pathological remodeling of the mature mitral valve. Indeed, elevation of leaflet stresses alone, imposed by papillary muscle tethering in adult sheep, triggered a 17% increase in leaflet area after 2 mo (12). In LV dysfunctions that involve orifice expansion, stresses on the valve leaflets are increased with their radius of curvature (by the law of Laplace) as more of the functional reserve of the leaflet is used and the coaptation area is decreased. As a result, leaflet length and area increase in parallel with annular diameter (9, 18). Together, the results of the present and previous studies support our hypothesis that leaflet enlargement occurs along
with the cardiac and orifice expansion during pregnancy, in a manner similar to leaflet enlargement in LV dysfunctions, at least those where chamber and orifice expansion take place. Such leaflet enlargement with mitral valve orifice expansion during pregnancy may serve to maintain coaptation and may explain the clinical absence of increased mitral regurgitation in the maternal circulation.

The increase in size of the bovine anterior mitral valve leaflet appears to be largely uniform across its area. We observed similar increases in both the radial and circumferential directions, with radial length increasing 20% and circumferential length increasing 14% from NP animals (Fig. 3, B and C).

In addition, the relative position and separation of the primary strut chordae were increased in step with the leaflet radial and circumferential lengths, suggesting a uniform enlargement of the leaflet, at least between the primary struts and the attachment edge. It is unclear how this leaflet enlargement compares with that in LV dysfunction. Chaput and coworkers (9) suggested that the valve enlarges primarily through expansion in the circumferential direction, whereas Timek and coworkers (52) attribute it to a radial lengthening of the mitral leaflet, mainly near the free edge.

We compared the mechanical and thermomechanical properties of anterior mitral valve leaflets from NP animals with those reported in previous studies, and, where possible, statistical comparisons were performed. Our biaxial mechanical data are shown in Table 2, along with data from other studies on porcine and ovine anterior mitral valve leaflets. Included are previous data obtained using biaxial testing similar to that of the present study (32), intact valves in a physiological flow loop (20, 48), valves tested in situ (10), and valves assessed in vivo (3). The latter study examined the effects of...
referential configuration on calculated mechanical parameters. Table 2 shows Amini et al.’s data (3), where the reference state was obtained from the excised, stress-free leaflet. In general, relatively larger deformations (stretch ratios and extensibility) were observed using in vitro biaxial testing versus measurements from the studies on intact valves in physiological flow loops, either in situ or in vivo (Table 2). These differences arise largely from the choice of reference state in the strain or stretch ratio calculation. In the present study and that of Liao et al., a nearly stress-free configuration was used as the reference state, where the square biaxial specimen was loaded only enough to hold the sample flat. The other studies (10, 20, 48), on porcine valves, used intact but unloaded leaflets (whether mounted in a flow loop or in situ) as a reference state, which reflects the in situ geometry and residual stresses in the leaflet.

Data from NP animals in the present study were somewhat comparable with those from porcine anterior leaflets under

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**Fig. 9.** Representative Verhoeff-van Gieson (VVG)-stained radial cross-sections of the anterior mitral valve leaflet showing histological changes with pregnancy and its duration. In VVG-stained tissue, black denotes elastic fibers and cell nuclei and purple-pink denotes collagen fibers. Proteoglycans/glycosaminoglycans are not stained. Full-thickness cross-sections are shown in A, E, I, and M, with the atrialis at the top of the image and the ventricularis at the bottom. B–D, F–H, J–L, and N–P show higher-magnification images of the atrialis, spongiosa, and ventricularis for each sample in A, E, I, and M. A: full-thickness image from a NP heifer; B–D: atrialis (B), spongiosa (C), and ventricularis (D), respectively, from the same tissue. E: full-thickness image from a cow in the EP group (113 days of gestation); F–H: atrialis (F), spongiosa (G), and ventricularis (H), respectively, from the same tissue. I: full-thickness image from a cow in the LP group (184 days of gestation); J–L: atrialis (J), spongiosa (K), and ventricularis (L), respectively, from the same tissue. M: full-thickness image from cow in the LP group (240 days of gestation, close to term); N–P: atrialis (N), spongiosa (O), and ventricularis (P), respectively, from the same tissue. Scale bars = 200 μm in A, E, I, and M; scale bars = 100 μm in B, F, J, and N and are representative for the remaining images.
biaxial testing. Our mean \( \lambda^{\text{peak}} \) (1.37 ± 0.05) was 23% larger (\( P = 0.003 \)) than that obtained for the porcine anterior leaflet (1.12 ± 0.05, \( n = 5 \)) (32), whereas there was no significant difference between our \( \lambda^{\text{peak}} \) (1.67 ± 0.06) and that obtained for porcine valves (1.26 ± 0.04, \( n = 5 \); Table 2). The slightly higher circumferential extensibility in the present study is likely due to species-related differences.

There was, however, excellent agreement between our data from NP animals and those from Animi et al.’s ovine in vivo model (3), which used the excised, stress-free configuration as the reference state (Table 2). There was no significant difference between our \( \lambda^{\text{peak}} \) (for NP animals) and that of Animi et al. (1.22 ± 0.07, \( n = 4 \)), and, similarly, no significant difference between our \( \lambda^{\text{peak}} \) and that of Animi et al. (1.65 ± 0.08, \( n = 4 \)).

The greater stretch ratios in both directions in the present study translate into a larger areal stretch, as observed from porcine leaflets assessed in a physiological flow loop (the only previous studies where areal strain is reported). Mean areal stretch from the present study (from NP animals) was 2.3, indicating an average area increase of 130% from the unloaded state to peak equibiaxial loading conditions. This is ~60% larger than values reported for porcine anterior leaflets (1.42–1.47; Table 2). These differences are likely a result of the differences in both species and referential conditions between the two studies.

Interestingly, the relative stretches in the two principle directions were remarkably similar across species and study conditions (Table 2). The ratio of peak circumferential to radial stretch ratios from NP animals in our study (0.82) was very similar to the same ratio observed in porcine valves under biaxial testing (0.86 (19)) and physiological flow loop (0.83), as well as from ovine valves assessed in vivo (0.89 (47)), suggesting that the loading conditions were quite consistent across testing protocols and species and are similar to conditions observed in vivo.

As for our thermomechanical data, values for NP animals from the present study (\( T_d = 68.6 \pm 0.5^\circ\text{C}, t_{1/2} \) in control = 8.6 ± 1.1 h) were slightly higher than values reported for adult steers (\( T_d = 67.1 \pm 0.50^\circ\text{C}, t_{1/2} \) in control = 3.2 ± 0.5 h (2)). These differences may be sex related, since the cattle studied in the present and previous study were from similar ages (12–24 mo in the present study and 24–30 mo in the previous study).

Finally, our changes in the bovine heart mass and dimensions are comparable with those from previous studies on LV remodeling during pregnancy in humans. The 45% increase in heart mass during bovine pregnancy was comparable with the 52% increase in LV mass in humans (45). Similarly, the 15% increase in heart circumference was comparable with the 12% increase in LV end-diastolic diameter in humans (24). These comparisons support the validity of our bovine model to study cardiovascular adaptations to pregnancy.

One of the most striking observations from the present study was that the leaflet dimensions and mechanical properties did not change along the same timeline (or direction) during pregnancy. This may suggest that different remodeling mechanisms are at play in EP versus LP. New leaflet dimensions are attained in EP, with leaflet area increasing by 33%. Accompanying this enlargement is a 30% decrease in leaflet extensibility, largely in the circumferential direction. EP may therefore represent a period when valve leaflets attain the new anatomic/dimensional “set point” associated with the enlarged valve orifices necessary in pregnancy. We do note, however, that all measurements of cardiac mass and dimensions in the present study were not significantly increased until LP. While we do not have direct measurements of valve orifice areas, a previous study (45) has suggested that their enlargement with pregnancy is progressive. Regardless of the trigger, the mechanism underlying the EP enlargement of the leaflet may be achieved by one or more factors, including 1) growth of new tissue, 2) plastic deformation (permanent stretch) of existing tissue, or 3) changes in collagen architecture. The simultaneous thicken-
ing of the leaflet in EP strongly suggests that tissue growth is occurring. Indeed, the transient increase in leaflet thickness in EP may underlie the shift to the left of the tension versus stretch curve. This may be followed by the mechanism proposed in heart failure, with plastic deformation of the tissue (17), as suggested by the subsequent thinning of the leaflet with apparent “elongation” of the fibrosa in the radial direction.

As for changes in collagen architecture, loss of collagen crimp could contribute to leaflet expansion in the circumferential direction; however, this appears to occur in LP, after the expansion has occurred.

In LP, whereas the enlarged leaflet dimensions are maintained, extensibility then increases (largely in the radial direction) back to prepregnant values by term. The trigger for this remodeling may be the increased stress in the leaflet that must accompany its enlargement. LP may then represent the phase where the valve leaflet becomes “entrenched” at these increased dimensions and, as it remodels toward normalizing leaflet stress, reattains its prepregnancy mechanical properties. It is interesting to note that this response of the mitral leaflet parallels that seen in the artery wall in response to chronic increases in blood flow (5, 29). Initially, the vessel diameter expands passively (in this case, with shear-induced vasodilation), achieving a larger-diameter lumen. The increased tensile stress (via the law of Laplace) triggers remodeling of the vessel wall, with hypertrophy of smooth muscle cells and increased extracellular matrix production. This stress-induced remodeling of the vessel wall has the dual effect of (1) entrenching the vessel at this new, larger diameter and (2) reattaining the native mechanical properties of the vessel (5). Such a mechanism may be at play here.

An understanding of the complex interplay between leaflet dimension and mechanical properties during mitral valve remodeling will require more detailed histological and biochemical analyses. Expansion of the leaflet with a biphasic change in extensibility could involve changes in leaflet thickness, collagen architecture, or even the intrinsic properties of the leaflet collagen itself (e.g., via cross-linking). Evidence suggests that all three of these factors are affected by pregnancy. Further studies will elucidate the contribution of these structural and material factors to the pregnancy-induced remodeling of the mitral leaflet. In particular, quantitative histological data on the biphasic changes in collagen crimp and leaflet thickness during pregnancy will allow us to examine their potential contributions to the biphasic changes in leaflet extensibility. In addition, biochemical and small-angle light scattering analyses are underway that will map pregnancy-related changes in composition and collagen fiber orientation over the entire leaflet area.

This study has also demonstrated, for the first time, an increase in the number of attachments of chordae tendineae on the mitral anterior leaflet as part of an adaptive remodeling response. This emphasizes that chordae, like leaflets, are dynamic, remodeling structures. This observation is in agreement with a previous study (42) that suggested that mitral valve chordae have fibroblasts capable of remodeling an extracellular matrix that appears, as in tendon, to be matched to physiological loading conditions. Increases in leaflet stresses during pregnancy would inevitably translate into elevated tensile stresses in the supporting chordae, triggering this interesting adaptive response of increased chordae attachments. Our observations raise questions regarding the mechanism underlying the increase in the number of chordal attachments during pregnancy-induced remodeling of the anterior leaflet. One possibility is that existing chordae split, beginning at their ventricular attachment site, as the leaflet surface expands. Further studies in our laboratory are investigating structural and mechanical alterations to the mitral valve chordae during pregnancy.

The increase in the number of chordal attachments during pregnancy did not quite keep in step with the increase in leaflet area. Thus, there is an overall decrease in chordae density during pregnancy that may result in further elevations of chordae stresses beyond those created by elevated leaflet stresses. This suggests that the process of increased chordae attachments (by chordal splitting or otherwise) may be unable to keep pace with the maternal cardiac adaptations to pregnancy (i.e., expansion of valve orifices and leaflet areas).

The remodeling of the mitral valve in pregnancy parallels some of the changes observed with pathological LV dysfunction. This is not surprising since many of the normal effects of pregnancy can resemble mild cardiac failure (56). Indeed, the cardiovascular changes associated with pregnancy may unmask preexisting heart conditions or precipitate new onset heart failure (21). It has been proposed that the altered cardiac structure and function in heart failure, which (as in pregnancy) leads to increased stresses on the mitral valve leaflets, induces “dysfunctional” remodeling of the tissue (17). In patients with functional mitral regurgitation due to LV dysfunction, mitral valve leaflets enlarge, lengthening along their midline (52), increasing leaflet area by up to 35% (9). In addition to these geometrical changes, leaflet composition (18, 26, 40) and mechanical properties are altered in heart failure, with leaflets becoming stiffer, less extensible, and less viscous (17). Grande-Allen and coworkers (17) proposed that in adaptation to LV dysfunction, mitral leaflets become permanently dis tended, thereby reducing extensibility and leading to decreased coaptation and mitral regurgitation. Thus, in these pathologies, the valve initially adapts to the increased size of the cardiac chambers and valve orifices, but with stiffened leaflets, this compensatory mechanism becomes insufficient, leading to mitral regurgitation (9, 14).

One important difference between human pregnancy and heart failure, however, is that mitral regurgitation becomes significant in heart failure (23) but not in pregnancy (7, 46). The results of the present study raise the intriguing possibility that the reversal of extensibility (i.e., an increase back to NP properties) of the enlarged leaflets in LP contributes to the maintenance of coaptation. This may be the mechanism under which mitral regurgitation is largely uncommon in pregnancy, despite the large increase in valve orifice area (7, 46). That is, with volume loading and valve orifice expansion, the mitral leaflet may not maintain coaptation simply due to its functional reserve but by adaptive remodeling and tissue growth that increases the size and alters the material properties of the leaflet. It is important to note, however, that we have no evidence of preserved mitral function in our bovine model, and we can only speculate that the same remodeling mechanism is at play in other species, including humans.

Further evidence for remodeling of the mitral leaflet during pregnancy was provided by our observation of a rapid decrease in the thermal stability of the tissue. In the present study, Tg of the anterior mitral leaflet was decreased, by over 2°C, in EP.

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While the magnitude of this decrease may seem small, it is on the order of changes (increases) in $T_d$ that are seen in cardiovascular tissues [in the pericardium (39) or heart valve leaflets (1)] during development, presumably as the collagen network matures and turnover decreases.

$T_d$ is a measure of the hydrothermal stability of the collagen triple helix and is influenced by number of factors, including the degree of intrahelical hydrogen bonding, intrahelical cross-linking, and molecular packing. The latter factor, as described by the polymer in a box model (37), influences the molecular stability of collagen through the packing and proximity of surrounding molecules and is suspected to underlie the changes in $T_d$ observed with hydration (36). Decreases in the thermal stability of collagen with mechanical loading conditions are also explained by this mechanism, where decreases in molecular packing (associated with increased collagen synthesis and turnover) are reflected by lower thermal stability of the tissue. In our previous study on perinatal heart valve development, we observed such changes in $T_d$ after birth, when mechanical loading changes differentially between the aortic and pulmonary valves. The relationship between thermal stability and mechanical loading conditions is also seen in the adult heart, where leaflet $T_d$ varies inversely with the maximum transvalvular pressure for that valve (2). While these studies can only infer about the state of the collagen network from its thermal stability, studies from Merryman and coworkers (34, 35) directly demonstrated the relationship between transvalvular pressure and the tissue remodeling state: collagen synthesis is higher and valvular interstitial cells have a “more pronounced ability to remodel valvular tissues” under higher transvalvular pressures. Thus, extending the polymer in a box model to our data suggests that the thermal stability of the mitral leaflet decreases as a result of the increased collagen remodeling and/or turnover that would be expected to accompany the increased mechanical loading conditions on this tissue during pregnancy.

In keeping with previous work, this study examined the ratio of immature-to-mature cross-linking as a proxy for the proportion of more recently synthesized collagen and, hence, the “remodeling state” of the tissue. Thus, a higher proportion of immature cross-links indicates a more rapid turnover rate of collagen (1, 4). Treatment of our tissues with NaBH$_4$ significantly increased HIT $t_{1/2}$ for tissues from all pregnancy groups, indicating the presence of thermally labile, immature cross-links. However, the magnitude of this increase was, surprisingly, unchanged across pregnancy groups, suggesting that the proportion of immature collagen cross-links remains similar with pregnancy. In addition, the relative increase in isothermal $t_{1/2}$ after NaBH$_4$ stabilization was small (increased by less than a factor of 2) compared with other studies [e.g., a 25-fold increase in the ovine pericardium during development (39)], suggesting that the content of immature cross-links remains relatively low throughout pregnancy. This was an extremely surprising observation. The present study has clearly shown that the anterior mitral valve leaflet undergoes rapid and complex alterations in its structure and mechanical properties during pregnancy. While this elevated remodeling state results in a lower thermal stability, as expected, it apparently does so with no change in the proportion of immature cross-linking.

One potential explanation for this observation is that we may have underestimated the relative proportion of immature collagen cross-linking using HIT with the NaBH$_4$ stabilization technique. This method uses the ratio of thermally labile to borohydride-stabilized collagen cross-links as an indicator of the ratio of immature to mature cross-links. As we have previously pointed out (1), these two ratios are not synonymous. While hydrothermally stable cross-links include mature cross-links HHL and PYD, they also include the intermediate immature cross-link DHLNL, leaving only the intermediate cross-link HLN from the truly hydrothermally labile cross-link. The thermal stability of the immature cross-link DHLNL would result in an underestimation of the immature-to-mature cross-link index assessed by HIT. It has recently been reported that valvular tissues contain relatively more ketoimine-derived cross-links (including the thermally stable intermediate DHLNL as well as PYD) than aldimeine-derived cross-links (HHL and HLN). Therefore, immature cross-links may indeed be present in large amounts in the mitral anterior leaflet, and may be increasing during pregnancy, but the majority of them may be of the DHLNL form and thus undetectable using our technique. Further studies on mitral leaflet collagen cross-linking during pregnancy must be carried out using direct HPLC techniques.

While this study clearly demonstrated rapid and significant remodeling of the mitral anterior leaflet during pregnancy, we were not able to separate the role(s) of hemodynamic stresses and hormonal changes as potential triggers of this remodeling. The relationship between pregnancy hormones and tissue remodeling is complex and difficult to define, given the potential influence of plasma hormone levels and the presence of hormone receptors in the tissue of interest and receptor sensitivity. Nonetheless, hormonal changes during pregnancy have demonstrable effects on the remodeling of both reproductive and nonreproductive tissues. Growth hormone (28), estrogen (6), and relaxin (50) can trigger tissue remodeling, modulating the deposition and/or degradation of the extracellular matrix. These pregnancy hormones, which target collagen (the predominant structural load-bearing component of heart valve leaflets), would be of particular interest.

In summary, we demonstrated a physiological, adaptive remodeling of the anterior mitral valve leaflet during the volume loading and cardiac expansion of bovine pregnancy. There was a rapid increase in leaflet area with a concomitant decrease in extensibility in EP, changes that parallel those in heart failure. There was also a surprising increase in the number chordal attachments that might accommodate the inevitable increases in chordal stresses. In LP, leaflet extensibility then increased, largely in the radial direction, back to its prepregnant value. This remodeling may serve to compensate for the increased physiological loading conditions associated with pregnancy by normalizing leaflet stress and maintaining coaptation. Understanding the mechanisms of mitral valve physiological remodeling in pregnancy could contribute to the development of alternative treatments of mitral valve pathological remodeling associated with LV dysfunction, where valve coaptation is not maintained. An understanding of these mechanisms will also be fundamental to the field of tissue engineering as it strives to direct the proper growth and function of heart valves and other load-bearing tissues.

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Author Contributions

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Disclosures

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References


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