Sex-dependent Remodeling of Right-Ventricular Function in a Rat Model of Pulmonary Arterial Hypertension

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ABSTRACT

Right-ventricular (RV) function is an important prognostic indicator for pulmonary arterial hypertension (PAH), a vasculopathy that primarily and disproportionally affects women with distinct pre- and post-menopausal clinical outcomes. However, most animal studies have overlooked the impact of sex and ovarian hormones on RV remodeling in PAH. Here, we combined invasive measurements of RV hemodynamics and morphology with computational models of RV biomechanics in sugen-hypoxia (SuHx) treated male, ovary-intact female, and ovariectomized female rats. Despite similar pressure overload levels, SuHx induced increases in end-diastolic elastance and passive myocardial stiffening, notably in male SuHx animals, corresponding to elevated diastolic intracellular calcium. Increases in end-systolic chamber elastance were largely explained by myocardial hypertrophy in male and ovary-intact female rats, whereas ovariectomized females exhibited contractility recruitment *via* calcium transient augmentation. Ovary-intact female rats primarily responded with hypertrophy, showing fewer myocardial mechanical alterations and less stiffening. These findings highlight sex-related RV remodeling differences in rats, affecting systolic and diastolic RV function in PAH.

NEW AND NOTEWORTHY

Combining hemodynamic and morphological measurements from male, female, and ovariectomized female PAH rats revealed distinct adaptation mechanisms despite similar pressure overload. Males showed the most diastolic stiffening. Ovariectomized females had enhanced myocyte contractility and calcium transient upregulation. Ovary-intact females primarily responded with hypertrophy, experiencing milder passive myocardial stiffening and no changes in myocyte shortening. These findings suggest potential sex-specific pathways in RV adaptation to PAH, with implications for targeted interventions.

INTRODUCTION

Right-ventricular (RV) function is an important predictor of long-term outcomes in pulmonary arterial hypertension (PAH), a severe vasculopathy that progresses to RV dysfunction and failure.[1,2] In PAH, sustained elevation of mean pulmonary arterial pressures exceeding 20 mmHg impose a pressure overload on the RV, causing ventricular remodeling. Preclinical studies of RV pressure overload reveal common changes, including hypertrophy, fibrosis, altered contractility, and increased myocardial stiffness.[3–7] Although RV hypertrophy can initially help to reduce wall stress and maintain cardiac function, these changes can lead to maladaptive functional alterations. Clinically, changes in RV diastolic stiffness and contractility have been closely associated with disease severity in patients with PAH.[8,9] A 10-week study on the progression of PAH in male sugen-hypoxia (SuHx) rats showed significant increases in RV end-diastolic pressures and chamber stiffness, peaking at 8 weeks following disease induction. These changes were primarily attributed to increased passive myocardial stiffness, rather than alterations in RV geometry.[10]

Hypertrophy and wall thickening appeared to stabilize systolic function in the early stages of the disease, despite subsequent mechanical changes.[5,10]

PAH disproportionally affects women, with pre-menopausal women exhibiting less severe RV remodeling and better cardiac outcomes than post-menopausal women or men.[11,12] Clinical[13–15] and preclinical studies[7] have highlighted the association between sex and RV hemodynamic function, with correlations found between improved RV function and endogenous or exogenous estrogens.[16,17] However, most animal studies have focused on male subjects, limiting sex-specific data on RV remodeling. As a result, the role of sex in PAH remains unclear. Estrogen may influence RV myocardial remodeling, explaining the sex-specific differences in disease progression.[7,16] Therefore, there is a critical need to understand the influence of sex on the pathological remodeling of the RV that occurs in PAH.

To address the knowledge gap in sex-specific RV remodeling, we conducted a study using hemodynamic and morphologic measurements, and a biomechanics model of the RV. We aimed to determine the effects of chamber geometry and myocardial material properties on end-systolic and end-diastolic pressure-volume (P-V) relations. Our study used the sugen-hypoxia (SuHx) model of PAH male, ovary-intact female, and ovariectomized (OVX) female rats using. We hypothesized that sex differences in RV organ-level performance would correspond with differences in myocardial material remodeling. Specifically, female SuHx-treated rats would show increased contractility with mild diastolic stiffening, contrasting with severe chamber stiffening in male SuHx-treated rats. Ovariectomized SuHx rats, lacking ovarian hormones, would display a more severe RV remodeling phenotype compared to ovary-intact females but less severe than males. Using RV P-V measurements, we predicted myocardial mechanics and validated these predictions with myocyte mechanics data.

METHODS

Animal model of PAH

The SuHx rat model of PAH was chosen for this study because it replicates vascular lesions similar to those observed in autopsies of patients with PAH, leading to subsequent RV remodeling.[18] Animal care, housing, and feeding protocols were approved by the Institutional Animal Care and Use Committee at the University of California San Diego. The study involved 56 male and 86 female Sprague-Dawley rats, including 38 female rats that underwent ovariectomy procedures (OVX, Charles River Laboratories, Hollister, CA, USA) at 6 weeks of age. Male rats (7 weeks of age), ovary-intact female rats (8 weeks of age) and OVX female rats (8 weeks of age), each weighing approximately 200 grams, were randomly assigned to control and SuHx groups. The SuHx groups received the vascular endothelial growth factor receptor antagonist, sugen (SU5416, S8442 MilliporeSigma, CAS Number 204005-46-9, PubChem Substance ID 24278606 Sigma-Aldrich, MO, USA), at a dose of 20 mg/kg, and were then subjected to chronic hypoxia (10% O_2)

for 3 weeks. Following this period, they were then returned to normoxia for 5 weeks. Age-matched normotensive animals were kept in normoxia throughout the study as control groups. Whenever possible, experiments were performed sequentially within each animal. However, in the case of myocyte studies this was not always feasible.

Invasive Right Ventricular Hemodynamics

Right ventricular blood pressure and volume were measured simultaneously during an open-chest procedure, following previously described methods.[5,10,19] In this terminal procedure, animals were continuously administered 2.5% isoflurane (MWI Veterinary Supply, USA, cat #502017) mixed with oxygen (100% O_2) *via* a nose cone. Following a tracheotomy, the animals were intubated and ventilated at a respiratory rate of 40-50 breaths per minute with tidal volume ranging from 1.7-4.2 mL, determined by the animal's weight. Body temperature and heart rate were continuously monitored throughout the procedure using a rectal probe (MicroTherma 2, ThermoWorks, UT, USA) and a 4-lead electrocardiogram. The isoflurane concentration was occasionally adjusted up to 1.5% to maintain a heartrate range between 280-360 beats per minute. P-V timeseries were recorded with a 1.9F admittance catheter (Transonic Scisense, Ontario, Canada)[20–23] which was inserted apically into the RV chamber. P-V data obtained during caval occlusion were used to determine end-systolic (ES) and end-diastolic (ED) chamber elastances. Data were collected using LabChart software (version 8 Pro, ADInstruments Inc., Colorado Springs, CO, USA) and analyzed offline using custom-written MATLAB code (version R2021a, MathWorks, Natick, MA, USA).

Right-Ventricular Tissue Measurements

After *in vivo* hemodynamic measurements, the animals were exsanguinated, and the heart flushed with ice-cooled phosphate-buffered saline (pH 7.4) consisting of 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2HPO_4 and heparin solution (USP 5000 units/mL, MWI Veterinary Supply, USA, cat #054255). The heart was excised, and the RV free wall, left ventricular (LV) free wall, and septum were isolated and weighed. The Fulton index (the ratio of RV mass to the sum of LV and septal masses) was calculated, while myocardial volumes were determined by converting excised masses at a density of 1.053 g/mL[24] and used as model inputs.

The excised RV myocardium near the outflow tract was immediately fixed in 10% formalin (Thermo Fisher Scientific, MA, Cat. No. SF100-4) for 48 hours at room temperature and then stored in 70% ethanol (Decon Laboratories, PA, Cat. No. V1001) at room temperature. The fixed RV samples were paraffin-embedded and sectioned transmurally. Myocyte hypertrophy was quantified using the semi-automated histological analysis Fiji package HeartJ.[25] Analysis was performed on H&E stained images captured at 40x magnification with a resolution of 4.6pixels/μm. Myocytes, nuclei, and capillaries were segmented using the MorphoLibJ Fiji plug-

in.[25] Myocyte hypertrophy was characterized with the minimum Feret diameter (the distance between two parallel lines enclosing the myocyte) to account for variations in myofiber alignment. An average of 351±54 cells per animal were measured.

RV free-wall myocardium designated for collagen quantification was flash-frozen and stored at -80°C. Total collagen content was measured using a hydroxyproline assay (QuickZyme Total Collagen Assay, QuickZyme Biosciences, Leiden, NL).[10] RV free wall tissue samples weighing 5-15 mg were prepared and assayed according to the manufacturer's protocol. Hydroxyproline residues were quantified using a microplate reader (Tecan M200, Tecan LifeSciences, CH) at 560 nm. Absorbances were calibrated with linear collagen standard curves, adjusted for sample dilution, and expressed as total collagen concentration.

Right-Ventricular Isolated Myocyte Measurements

In separate groups of male, female, and OVX female rats (n = 11-13 per control group, n = 8-13 per SuHx group), RV pressure measurements were first obtained to confirm hypertension. The heart was then excised and mounted on a Langendorff apparatus, where the coronary vasculature was perfused with calcium-free Tyrode's solution (0.134 M NaCl, 0.00268 M KCl, 0.00556 M glucose, 0.0119 M $NaHCO_3$), followed by an enzymatic digestion solution (0.001 M collagenase type II, 0.0015 M protease).[26,27] After digestion, calcium was gradually reintroduced by perfusing Tyrode's solution with three serial dilutions of $CaCl_2$ up to 1.8 mM. The RV was isolated and separated from the septum, finely chopped, and manually agitated to dissociate individual cells.

The RV myocytes were then incubated in the dark at room temperature for 30 minutes in Tyrode's solution containing 2 μM of Fura 2-AM (Invitrogen, Thermo Fisher). The myocytes were subsequently seeded onto a microscopy platform and continuously perfused with Tyrode's solution containing 1.8 mM [Ca^{2+}], while being paced at a frequency of 1 Hz. A laser scanning fluorescent photometer (Calcium and Contractility System, IonOptix, MA, USA) equipped with a 40x immersion objective was used to record fluorescence excitation wavelengths of 340 nm and 380 nm.[28] Intracellular calcium timeseries and myocyte shortening measurements were recorded using IonWizard software (IonOptix, version 6.6).

Only rod-shaped cells with clear striations, which remained quiescent without electrical stimulation and exhibited no sarcolemmal blebbing were included. Continuous timeseries of sarcomere length and fluorescence intensity lasting 10-15 seconds were recorded during pacing. For each animal, 7-15 myocytes were analyzed. Cardiomyocyte contractile shortening was measured as the difference between diastolic sarcomere length at rest and peak sarcomere length during contraction. Fluorescence intensity signal was normalized by dividing the peak fluorescent intensity (F) by the average resting fluorescence (F_o)after background subtraction.as the ratio F/F_o was used to represent the cytosolic calcium concentration.

Biomechanics Model of the Right Ventricle

To assess how changes in RV chamber volumes and pressures in SuHx animals were influenced by alterations in RV wall geometry, as well as passive and active myocardial material properties, we used a previously described mathematical model of RV biomechanics.[5] This model represents the RV free-wall as a truncated portion of a sphere. RV wall stress was calculated using the Laplace's law, and end-diastolic myocardial wall tension was characterized by an exponential stress-strain relation.[29,30] Active systolic tension was determined based on empirical functions of sarcomere length, time, and intracellular calcium concentration.[31,32] Parameters such as sarcomere slack length (1.85 μm), maximal isometric tension (2666 kPa), maximal intracellular calcium concentration (4.35 μm), zero-stress sarcomere length (1.6 μm), and the peak isometric tension-length shape parameter B (12 μm^{-1}) were set based on previously reported values.[33] The distribution of end-diastolic volumes during caval occlusion was tight, leading to the averaging of ED P-V relations by volume (shown with horizontal error bars) and ES P-V relations by pressure (shown with vertical error bars). The passive material stiffness parameters $\{k_1, k_2\}$ were fitted to predict ED volume from ED pressure measurements during vena cava occlusions. The third material parameter k_3 (representing contractility) was then fitted to predict ES pressure from ES volume measurements during vena cava occlusions. The root-mean squared error (RMSE) between predictions and measurements was the minimization metric.

Statistical Analysis

Group comparisons of hemodynamics, morphological measurements, myocyte mechanics, and model-derived results were analyzed with descriptive statistics in JMP Pro Statistical software (version 16, SAS Institute Inc., NC, USA). Two-factor ANOVA was used for normally distributed data to evaluate differences due to treatment (control vs. SuHx) and animal group (male, ovary-intact female, and ovariectomized female). Post-hoc comparisons were performed using the Dunnett's test for treatment comparisons and the Tukey test for animal group differences. Following two-factor ANOVA, simple main effects were analyzed with the F-statistic for group and treatment. Interaction effects between group and treatment were reported as ($F_{\text{df-a,df-b}}$ = $F_{\text{interaction}}$, p-value), where df-a and df-b refer to the degrees of freedom of each factor (treatment, group), and $F_{\text{interaction}}$ is the ratio of interaction mean squares to mean squared error. For non-parametric data, the Wilcoxon-Kruskal-Wallis statistic was used, followed by the Dunnett's test for treatment comparisons and the Dunn test for animal group comparisons. Rat-specific sarcomere mechanics predictions were analyzed using a general linear model with mixed factors to compare treatment (inter-group) and sarcomere length (inter-level) effects. For fixed factors, two-factor ANCOVA and analysis of means were applied to assess the means and slopes of sarcomere length for ED-derived data, and fiber stress for ES-derived data. Statistical significance was set at $\alpha = 0.05$. All figures were generated using Prism software (version 8.4.3, GraphPad, CA, USA).

RESULTS

Effects due to SuHx

Pulmonary arterial hypertension was successfully induced in 96% of the rats (defined as SuHx-treated rats with mean pulmonary arterial pressures >20 mmHg), regardless of sex. SuHx rats exhibited significantly higher mean pulmonary arterial ($p < 0.001$) and RV end-systolic ($p < 0.001$) and end-diastolic ($p < 0.001$) pressures compared to their control groups (**Figure 1A-B**). After normalizing the excised RV mass by the sum of LV and septum mass, the Fulton index showed similar increases in all SuHx animals. Specifically, the Fulton index increased from 0.26-0.28 in control groups to 0.53-0.55 in SuHx groups ($p < 0.001$, **Figure 1C**). RV end-systolic volume (**Figure 1D**) increased significantly in SuHx rats ($p < 0.001$). End-diastolic volumes (**Figure 1E)** were largely unchanged by SuHx ($p = 0.068$), however there was a significant interaction between treatment and group effects ($F_{1,2} = 4.75$, $p = 0.012$). Post-hoc analysis revealed a significant increase in ED volume only between the OVX control and SuHx group ($p < 0.001$).

No significant effects of SuHx on stroke volume (**Figure 1F**) were found ($p = 0.35$). End-systolic elastance (E_{es}) significantly increased in SuHx animals ($p < 0.001$, **Figure 1G**). For end-diastolic elastance (E_{ed}, **Figure 1H**), a significant interaction effect was observed between treatment and group ($F_{1,2} = 11.98$, $p < 0.001$) and the main effects analysis showed that ED elastance significantly increased in SuHx animals ($p < 0.001$).

Ejection fraction (**Figure 1I**) significantly decreased in SuHx rats ($p = 0.0012$), falling from 63.7% to 50.9% in male SuHx, 58.4% to 52.7% in female SuHx, and 60.1% to 51.9% in OVX SuHx groups, with no decline exceeding 12.8% in any group. SuHx treatment resulted in significant increases in mean pulmonary arterial pressure (mPAP, $p < 0.001$, **Figure 1J**) and arterial elastance (E_a, $p < 0.001$, **Figure 1K**). However, the E_{es} to E_a ratio (ventricular-vascular coupling, **Figure 1L**) did not change due to SuHx ($p = 0.21$), indicating no changes in right-ventricular pulmonary-arterial coupling.

Effects due to sex

There were no significant differences in RV ES pressures ($p = 0.91$, **Figure 1A**) or Fulton Index ($p = 0.44$, **Figure 1C**) between the three animal groups. While ED pressures (**Figure 1B**) increased in all SuHx animals ($p < 0.001$), no significant interactions between group and treatment were found ($F_{1,2} = 1.63$, $p = 0.20$). However, we note male SuHx rats exhibited a six-fold increase, compared to a two-fold increase in female SuHx and a three-fold increase in OVX SuHx rats. Male control rats had mean pulmonary arterial pressures of 16.9±0.4 mmHg, compared to 15.3±0.8 mmHg in the female group and 15.4±0.7 mmHg in the OVX controls. SuHx treatment increased mPAP to 39.1±4.0 mmHg in males, 42.7±3.8 mmHg in females, and 42.8±3.3 mmHg in OVX females. Two-factor ANOVA revealed no significant effects of group ($p = 0.87$) nor significant interactions between group and treatment ($F_{1,2} = 0.76$, $p = 0.47$).

RV ES volume (**Figure 1D**) increased significantly in all SuHx-treated animals ($p < 0.001$), but no significant group effects on ES volume were found ($p = 0.64$), nor were there significant interactions between group and treatment ($F_{1,2} = 2.57$, $p = 0.084$). It is noteworthy that the mean ES volume was greater in the OVX SuHx group than in the male or female SuHx groups. Two-factor ANOVA revealed significant interactions between treatment and group on ED volume ($F_{1,2} = 4.75$, $p = 0.012$, **Figure 1E**). Post-hoc analysis showed a significant increase in ED volume in the OVX group (37% increase, $p < 0.001$ compared with OVX controls), but not in the female group (6% increase, $p = 0.85$) or the male group (3% decrease, $p = 0.99$). There were no significant effects of treatment ($p = 0.35$), group ($p = 0.98$), or interactions between treatment and group ($F_{1,2} = 2.86$, $p = 0.0647$) on RV stroke volume (**Figure 1F**). No significant group effects on ejection fraction were observed ($p = 0.89$, **Figure 1I**), nor were there significant interaction effects between treatment and group ($F_{1,2} = 0.51$, $p = 0.60$). However, the largest mean decrease in ejection fraction was observed in the male rats (from 63.7% to 50.9%), followed by OVX rats (from 60.1% to 51.9%) and female rats (from 58.4% to 52.7%).

Both ES and ED chamber elastance (**Figure 1G-H**) significantly increased in SuHx animals, with the largest increases observed in male SuHx. Significant effects due to animal group were found for ED elastance ($p < 0.001$), with significant interaction effects between group and treatment ($F_{1,2} = 11.98$, $p < 0.001$). Post-hoc analysis revealed that ED elastance was higher in male SuHx rats compared to female SuHx ($p = 0.002$) or OVX SuHx rats ($p = 0.0194$).

Remodeling of the P-V relationships varied between SuHx groups (**Figure 2**). RV ES pressures were elevated to similar levels in all SuHx groups, but RV ES volume increased the most in the OVX group. While increases in end-diastolic pressure were comparable in male and OVX SuHx groups, RV chamber volume was preserved in male SuHx rats. Increases in the ED chamber volume tempered the steepening of the ED P-V relationship in the OVX SuHx group. In ovary-intact female animals, SuHx led to the smallest diastolic pressure-volume changes.

Model predictions of the relative contributions of geometry and wall properties due to SuHx

The model was initially used to assess the relative contributions of geometry and material properties to changes in systolic and diastolic P-V measurements. Increases in end-systolic pressures (**Figure 3A**) were primarily attributed to RV wall hypertrophy (geometry) in both male and ovary-intact female groups, with statistically insignificant contributions ($p = 0.34$) from changes in myocardial wall material properties. In the OVX group, significant increases in end-systolic pressure were attributed to both geometry and systolic material properties ($p = 0.029$). The rise in end-diastolic pressure (**Figure 3B**) in male SuHx rats aligns with previously documented alterations in myocardial wall properties.[10] Conversely, in ovary-intact female SuHx rats, the elevated diastolic pressure was primarily attributed to geometric changes. Notably, in the OVX SuHx group, both geometric and material property changes significantly contributed to the

increase in diastolic pressure, collectively explaining the observed rise. The greatest increases in ED pressure were observed in male SuHx rats, while the smallest were in female SuHx rats, reflecting the largest and smallest contributions from material property changes, respectively.

Thus, while changes in ES pressures among the hypertensive animals were primarily due to RV hypertrophy with significant contributions from systolic material changes in the OVX group, increases in ED pressures were associated with material stiffening in male and OVX SuHx rats which were most pronounced in males and to hypertrophy in female SuHx.

Model predicts most severe myocardial passive stiffening in male SuHx rats and recruitment of contractility in OVX SuHx rats

The model was then used to predict myocardial stress-strain relations. The mean passive and active sarcomere stress-length relations were derived using the model parameters k_1, k_2 and k_3, which were fitted to the end-systolic and end-diastolic P-V relations (**Figure 4**). The model predicted a significant increase in the passive stress-sarcomere length relation due to SuHx ($p < 0.001$, **Figure 4**), with the most pronounced stiffening observed in the male SuHx group (**Figure 4A**). While the slope of the active stress-sarcomere length relation increased due to SuHx in all groups ($p < 0.001$), only the ovariectomized group exhibited a significant rise in their active stress-sarcomere length relations (indicating increased myocardial contractility) compared to their respective control group ($p = 0.0133$, **Figure 4C**).

RV tissue shows myocyte hypertrophy and fibrosis in all SuHx groups

Myocyte diameter (**Figure 5A**) increased with SuHx in all groups. A two-factor ANOVA, followed by a main effects analysis showed a significant effect due to treatment ($p < 0.001$) and group ($p = 0.0179$), with significant interactions between treatment and group ($F_{1,2} = 27.73$, $p < 0.001$). Post-hoc analysis revealed a significant difference between the female and OVX groups ($p = 0.0173$). A two-factor ANOVA revealed a significant increase in collagen content (**Figure 5B**) due to treatment ($p < 0.001$), but not group ($p = 0.23$), with no significant interaction effects between treatment and group ($F_{1,2} = 0.91$, $p = 0.41$).

RV myocyte response to SuHx is sex dependent

Isolated right ventricular myocytes from OVX female SuHx rats showed a significant increase in sarcomere shortening (**Figure 6D**, 2.6%, $p = 0.003$). However, no significant changes were observed in the male (0.9%) or ovary-intact female SuHx groups (0.4% respectively). Two-factor ANOVA revealed significant differences between the animal groups ($p = 0.028$), but not due to treatment ($p = 0.072$), with no significant interaction effects ($F_{1,2} = 0.83$, $p = 0.44$).

In male SuHx rats, changes in intracellular calcium concentration were more prominent at diastole ($p < 0.001$*)* rather than at peak ($p = 0.089$*)* calcium concentrations, resulting in a net decrease in the calcium transient amplitude (**Figure 6E,** $p = 0.17$). Despite this decrease and the considerable increase to diastolic calcium in SuHx-treated male rats (**Figure 6A**), sarcomere shortening remained elevated.

In both the female ($p = 0.08$) and OVX ($p = 0.37$) SuHx groups, the calcium transient amplitude remained unchanged. However, the OVX group showed the most pronounced increase in peak intracellular calcium ($p = .0467$, **Figure 6C**), which corresponded to the largest increase in shortening ($p = .0027$). In ovary-intact female rats, changes due to SuHx in both diastolic and peak intracellular calcium were small ($p = 0.44$), the calcium transient shape remained largely unchanged, and shortening was maintained ($p = 0.29$, **Figure 6B**).

Amplitude-normalized calcium transients showed no significant differences in the shape across groups (not shown). There were no significant effects of treatment on the $T_{75\%}$ calcium relaxation time ($p = 0.44$), although the OVX group showed prolonged relaxation compared to the male ($p = 0.02$) or ovary-intact female groups ($p < 0.001$, **Figure 6F**).

DISCUSSION

In this study, we investigated the impact of sex and the depletion of ovarian hormones on RV remodeling in pulmonary hypertensive rats. After eight weeks of SuHx, all three groups exhibited similar increases in mean pulmonary arterial pressures, end-systolic RV chamber pressures, and RV hypertrophy. Consistent with previous observations,[10,34]RV hypertrophic wall thickening was identified as an adaptive remodeling response, contributing to the increased end-systolic chamber elastance in both male and ovary-intact female groups. However, the adaptation of the myocardial mechanical properties in response to pressure overload varied depending on sex and the presence or absence of ovaries. In male rats, the RV maintained its end-diastolic volume but with a severe increase in passive myocardial stiffness. Despite a decrease in the calcium transient amplitude and a reduced sarcoplasmic reticulum (SR) calcium load, which was accompanied by an increase in diastolic intracellular calcium, sarcomere shortening remained unaffected. SuHx female rats exhibited the smallest reduction in ejection fraction. Although they showed RV hypertrophy with small increases in stiffness and diastolic volume, there were no changes in contractility recruitment, sarcomere shortening, or calcium transients. In the ovariectomized female rats, SuHx resulted in a significant increase in RV end-diastolic volume. The model predicted significant myofiber contractility recruitment in the OVX SuHx group, confirmed *ex vivo* in isolated myocytes. The OVX SuHx rats showed large increases in sarcomere shortening, peak intracellular calcium, and calcium transient amplitude compared to the other groups.

Sex differences in hemodynamics and hypertrophy

Although the incidence of PAH is influenced by sex, several studies have reported similar pulmonary vascular hemodynamics between men and women following diagnosis. Multiple cohort studies of PAH[35–37] and chronic thromboembolic pulmonary hypertension (CTEPH)[15] have consistently shown no significant sex differences in mean pulmonary arterial pressure (mPAP) or pulmonary vascular resistance (PVR). Similarly, large-scale studies in US military veterans found no differences in mPAP between men and women with PAH, although women had a higher average pulmonary vascular resistance at diagnosis.[38] Our findings align with these reports, showing only minor differences in mPAP and PVR between male and female rats. However, some PAH studies in younger adults have observed sex differences in mPAP, with young men exhibiting higher mPAP and hemodynamic burden than young women.[35,39,40] These discrepancies may be due to differences in disease stage between preclinical models and studies focusing on patients near heart failure. Age-related changes in sex-hormone concentration may significantly affect PAH risk and severity. Previous studies have demonstrated that sex differences in mPAP diminish by age 45 to 65,[40,41] suggesting that the relationship between sex and hemodynamics is not only influenced by ovarian hormone presence (e.g. menopause), but also by age and age-related comorbidities.

In patients with PAH, RV mass hypertrophy varies by sex, with greater hypertrophy in men, and decreases with age in both men and women with PAH.[37,42] Our study confirms RV myocyte hypertrophy, but we did not observe significant differences in acute RV hypertrophy (Fulton Index) between groups or in myocyte diameter between males and females after eight weeks of SuHx treatment. Further research is needed to better understand the interplay between sex, age, and PAH.

Systolic function in patients

Several studies have indicated that women with PAH often exhibit a less pronounced reduction in ejection fraction compared to men with PAH, even with similar RV afterload.[13,43] In our study, no significant differences in ejection fraction were found between groups, although male and OVX SuHx groups showed a decline in ejection fraction that was 1.5 times greater (over 2 times in the male group) greater than the decline observed in the female group. However, none of the SuHx groups had an ejection fraction below 50%. Changes to ejection fraction observed in this study are consistent with previous reports in similar male rat SuHx models.[19,44] Although female patients with PAH may tolerate RV pressure overload for a longer duration, they may experience more severe RV impairment at the end-stage of the disease,[37] as observed in a Dutch PAH cohort by van Wezenbeek *et al*.. Interestingly, post-menopausal women in this cohort demonstrated preserved RV function prior to adverse events, indicating that this prolonged period of tolerated RV pressure-overload may occur independently of menopausal status, although this finding may be confounded by other age-related comorbidities. Tello *et al.* recently investigated sex differences in ventricular-vascular coupling in patients with PAH and found that women with PAH had improved ventricular-vascular coupling than men with PAH, despite similar RV afterload parameters,

pulmonary arterial pressure and resistance, and RV chamber volumes.[45] Similarly, sex differences in survival were linked to improved systolic function and RV-PA coupling in women with CTEPH.[46] In our study, no differences in ventricular-vascular coupling were observed between groups or due to SuHx, and the E_{es}/E_a ratio remained within the healthy coupling ratio of 1.1-1.5.[47] Nevertheless, our findings are consistent with clinical reports, highlighting sex-dependent RV adaptations that occur independently of RV afterload.

Diastolic function in patients

RV diastolic stiffening (E_{ed} increase) is known to be a strong prognostic indicator for patients with PAH.[8,48] However, the relationship between diastolic stiffening and systolic output is complex and influenced by various factors such as RV loading conditions, RV wall thickness, myocardial fibrosis, and myocyte properties. Studies by Trip *et al.* and Vanderpool *et al.* have emphasized the importance of E_{ed} as an early predictor of therapeutic response in advanced PAH and as a potential mediator of age-related differences in PAH prognosis.[48,49] E_{ed} alterations are suggested to improve stroke volume by restoring the Frank-Starling volume reserve independently of contractility recruitment.[49,50] Additionally, diastolic stiffening may serve as an adaptive mechanism that helps prevent or delay RV dilation and dysfunction.[10]

However, the relationship between sex and diastolic stiffening in the RVs of patients with PAH remains unclear. Previous studies investigating diastolic stiffening in patients with PAH either did not examine sex differences or found no significant differences.[8,37,48,49] In our study, we observed increased end-diastolic chamber elastance in male and female (both ovary-intact and OVX) SuHx rats, with the most severe myocardial stiffening in male SuHx rats. Thus, we provide novel evidence of sex differences in RV diastolic stiffening and myocardial passive diastolic stiffening in the remodeled RV.

When the RV myocardium is tested *ex vivo* via planar biaxial loading, significant stiffening was found in SuHx-treated animals, with the most pronounced stiffening in males and the least in females.[51,52] Conversely, Cheng *et al*. reported diastolic stiffening without sex differences in a pressure overload murine model.[53] These discrepancies may be attributed to the specific animal model or stage of the disease. It is very likely that diastolic stiffening in PAH is a dynamic process that evolves over time, even during the early-stage of compensated RV remodeling.[10] Therefore, additional studies on diastolic function in PAH, particularly with respect to age and sex, are warranted.

Myocyte mechanics

While our study revealed greater myocyte shortening compared to some studies using similar isolated preparations,[22,27,54] our findings on myocyte contraction and calcium transients are in agreement with other previous studies and within physiological ranges.[55–57] Our results align with

previous measurements of field-stimulated rat ventricular myocytes, which showed greater myocyte shortening in young adult males compared to age-matched females.[55–57] It is well-documented that myocyte Ca^{2+}-dependent active tension increases early in PAH and remains elevated before the onset of RV decompensation.[8,58,59] During decompensation, myocyte peak tension is often downregulated due to intrinsic contractile protein dysfunction or other changes.[58,60,61] However, the progression and relationship between myocyte contractility and organ-level RV compensation remains unclear. Our study did not find evidence of RV decompensation or reduced contractility but did reveal sex and ovarian-hormone dependent changes in myocyte contractility.

In male SuHx rats, diastolic intracellular calcium increased significantly in male SuHx with a corresponding decrease in calcium transient amplitude. This finding aligns with previous reports showing reduced RV myocyte calcium transients in experimental models of severe RV hypertrophy,[62,63] as well as previous reports that male rat cardiomyocytes have higher diastolic calcium concentrations than females.[55,56] Previous studies have attributed impaired intracellular calcium removal to decreased sarco/endo-plasmic reticulum Ca^{2+} ATPase SERCA expression[64,65] or ryanodine receptor calcium leakage.[66–70] However, these differences have not been found between male and female rats at baseline.[71–73] Our data suggest that impairment of SR Ca^{2+} load may be a characteristic of this stage of male SuHx, as seen in left heart failure.[74] The increase in diastolic intracellular calcium may contribute to the dysregulation of excitation-contraction coupling and progressive contractility downregulation observed in male PAH.[9] This finding aligns with evidence that β_1–adrenoceptor blockade delays the onset of right heart failure by mitigating excitation-contraction coupling defects.[54,75] Interestingly, male SuHx-derived myocytes maintained their shortening despite reduced calcium transient amplitude, suggesting potential upregulation of myofilament calcium sensitivity, as previously reported in PAH.[76,77] However, this phenomenon was not observed in the female groups. Calcium hypersensitivity has been shown in OVX female rats but was inhibited by exogenous estrogen repletion.[78–80] It is possible that calcium hypersensitivity is present in both the male and OVX groups but is masked by calcium transient increases in OVX SuHx, as noted in previous reports. Changes in myocardial diastolic stiffness and myocyte calcium transient remodeling in male SuHx animals may act as complementary adaptive mechanisms to stabilize ejection fraction or may lead to progressive diastolic dysfunction. Further research into of sex-specific alterations in myocyte function are warranted.

LIMITATIONS

In this experimental design, sex refers to the biological sex of Sprague Dawley rats. We included ovariectomized female rats to simulate an ovarian-hormone-depleted RV remodeling phenotype, corresponding to the post-menopausal stage in women. It remains unclear why a small percentage of SuHx-treated animals (4% in this study) did not develop hypertension. Our ongoing research

includes studying potential PH biomarkers through lung histology and plasma analysis, but these normotensive animals were excluded from the current study. The animals used in this study were relatively young (~16 weeks old), which does not account for the effects of aging. Nevertheless, considering the pronounced effects of SuHx and their resemblance to features observed in humans, we believe this approach is justifiable. Ovariectomy procedures occurred prior to SuHx treatment and complete ovarian removal was confirmed during terminal endpoints. However, circulating hormone levels vary based on the estrous cycle stage, which was not controlled or tracked in this study. These hormonal fluctuations could impact sex-dependent alterations in ventricular mechanics.[81] However, the effects due to the estrous cycle are likely to be smaller than the variation caused by SuHx treatment and differences between animal groups. The admittance catheter used was calibrated with saline solutions of known conductivities suitable for blood and myocardium, a method validated against cardiac MRI for measuring RV volume in normotensive and hypertensive rodents.[82] While previous reports have shown that RV volumes measured *in vivo* using cardiac MRI tend to be smaller than those measured in open-chest preparations, ejection fraction remains consistent between noninvasive and admittance catheter-based measurements.[10] Although our results reveal sex-dependent remodeling of calcium-dependent myocyte contraction, the specific mechanism underlying this process remains unclear. Further studies are warranted to validate these findings, including research into the sex-dependent regulation, synthesis, and phosphorylation of sarcoplasmic ryanodine receptors, SERCA, and phospholamban under pressure overload conditions.

CONCLUSION

By combining right-ventricular hemodynamic and morphologic measurements with computational models of right-ventricular mechanics, we identified sex-specific differences in end-systolic and end-diastolic chamber function. Despite similar pressure overload from SuHx across groups, each group exhibited distinct RV changes. Passive myocardial stiffening was most severe in the male SuHx group, corresponding to an increase in diastolic intracellular calcium. In male and ovary-intact female rats, ventricular hypertrophy primarily explained the increases in end-systolic chamber elastance. In contrast, ovariectomized female rats recruited myofiber contractility recruitment *via* calcium transient enhancement. Ovary-intact female rats responded to pulmonary hypertension mainly through hypertrophy with fewer alterations in myocardial mechanics and the least passive stiffening. This difference may explain their improved long-term RV function and health outcomes.

AUTHOR CONTRIBUTIONS

EDK, BAH, TMW, DVJ - Conceived and designed research; EDK, BAH, KMG, HM, TMW, DVJ - Performed experiments; EDK, KMG, DVJ - Analyzed data; EDK, KMG, DVJ - Interpreted results and ran statistical analysis of experiments and models; EDK, KMG, DVJ - Prepared figures;

EDK, DVJ -Drafted and edited manuscript; EDK, BAH, KMG, HM, TMW, DVJ - Revised and approved manuscript.

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FIGURE LEGENDS

Figure 1. Invasive hemodynamic measurements of RV function in normotensive (control, solid) and hypertensive (SuHx, patterned) rats from male (grey), ovary-intact female (female, blue) and ovariectomized female (OVX, green) rats. There are significant increases in right-ventricular end-systolic pressure (**A**), end-diastolic pressure (**B**), and Fulton Index (**C**) across all SuHx-treated groups. End-systolic volume (**D**) notably increased in SuHx rats, while end-diastolic volume (**E**) only increased in the ovariectomized female rats. Stroke volume (**F**) remained comparable among groups. Both end-systolic (**G**) and end-diastolic (**H**) elastance increased with SuHx treatment, with the greatest rise observed in males. RV ejection fraction (**I**) decreased significantly due to SuHx. Mean pulmonary arterial pressure (**J**) and arterial elastance (**K**) also increased with SuHx treatment, irrespective of group. RV end-systolic elastance and arterial elastance remained coupled across all groups (**L**). #$p < 0.05$ effect of SuHx compared with controls, *$p < 0.05$ difference between animal groups.

Figure 2. Mean end-systolic (top) and end-diastolic (bottom) pressure-volume measurements for control (black) and SuHx (teal) male (**A**), ovary-intact female (**B**), and OVX female (**C**) rats during preload variation. Data shown as mean ± standard error.

Figure 3. The model discriminates the relative contributions of changes in RV geometry (patterned black) and myocardial material properties (patterned teal) in measured ED and ES pressure in control (solid black) vs SuHx (solid teal) groups. Increases in ES pressure (**A**) were largely explained by wall hypertrophy in both male and female rats with contributions from contractility recruitment that were significant in OVX female rats. Increases in ED pressures (**B**) were due primarily to myocardial material stiffening in male rats. Conversely, geometric remodeling was a contributor in both ovary-intact and OVX female rat groups in addition to material properties, but stiffening was much less pronounced in ovary-intact females. Data are shown as mean ± standard error. *$p < 0.05$ models compared with mean control group pressures. #$p < 0.05$ models compared with mean SuHx group pressures. Model predictions were based on control (10 male, 10 female, and 8 OVX) and SuHx (8 male, 13 female, and 10 OVX) rats.

Figure 4. Model predicted sarcomere length-tension relations in control (black) and SuHx (teal) animals shown as mean ± standard error of the mean. The model predicted significant stiffening in passive myocardial wall properties in SuHx-treated male (**A**), female (**B**), and ovariectomized female rats (**C**) with the most severe stiffening in male SuHx. While the slopes of the active length-tension relation increased in all SuHx groups, only the OVX group showed significant differences in active tension. *$p < 0.05$ compared with control group. Model predictions were based on control (10 male, 10 female, and 8 OVX) and SuHx (8 male, 13 female, and 10 OVX) rats.

Figure 5. H&E stained sections of the RV free wall show an enlarged myocyte diameter in SuHx-treated rats compared to the control group (A). In a hydroxyproline collagen assay, RV collagen content increased in all SuHx-treated groups (B). Data are shown as mean ± standard error. #$p < 0.05$ compared with control group. *$p < 0.05$ difference between animal groups.

Figure 6. Calcium transients (**A-C**) in isolated RV myocytes derived from male (grey), ovary-intact female (blue), and ovariectomized female (green) normotensive (solid) compared with SuHx (patterned) rats. SuHx treatment increased diastolic calcium concentration in males and OVX females, and intracellular calcium in OVX females. Sarcomere shortening (**D**) increased in the OVX SuHx group, while no significant changes were observed in the male or female groups. While SuHx caused no significant changes to calcium transient amplitude (**E**), the most pronounced changes to amplitude and to peak calcium concentration (**C**) were in the OVX group. While the time to 75% reduction from peak Ca^{2+} (T_{75}) was prolonged in the OVX group compared to both male and female groups, no significant differences were observed due to SuHx treatment (**F**). *$p < 0.05$ between groups. #$p < 0.05$ compared with control group. Data shown as mean ± standard error. Measurements obtained from control (11 male, 13 female, and 12 OVX) and SuHx (11 male, 9 female, and 13 OVX) rats.