Gender differences in left ventricular remodeling in chronic severe aortic valve regurgitation in rats.

Short title: Gender specific remodeling in AR

Keywords: female, rat, aortic valve regurgitation, remodelling, echocardiography

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Substantive abstract:

Background and aims: Aortic valve regurgitation (AR) can result in heart failure from chronic overloading of the left ventricle (LV). However, little is known about gender specific responses of the LV to this disease. We therefore compared LV remodeling between male and female rats with severe AR. To assess the impacts of estrogens on LV remodeling in AR, the effect of ovariectomy (OVX) was also evaluated.

Methods: AR was created in adults Wistar rats (females (control or OVX) and males). Animals were followed for 26 weeks and compared to sham-operated groups. Hearts were evaluated *in vivo* by echocardiography and harvested at the end of the protocol for tissue analysis.

Results: LV ejection fraction decreased similarly in both sexes. Despite similar echocardiographic AR severity, females had higher indexed cardiac output and the largest increase in LV weight, cardiomyocyte hypertrophy and eccentric remodeling. No differences were observed between ctrl and OVX females. Ovariectomy had no significant impact on any parameters.

Conclusion: Females developed more LV remodeling in response to chronic AR than male rats. AR seems to impose a greater LV workload on females due to their smaller body and heart size. Hormonal status did not have any impact on LV remodeling in this experimental model.

Short abstract:

Little is known about the gender specific adaptations of the left ventricle to chronic aortic valve regurgitation. We studied female and male rats with severe aortic regurgitation and also assessed the effects of ovariectomy in the females. Animals were followed for 26 weeks by echocardiography and hearts were harvested at the end of the protocol for tissue analysis. Female rats developed relatively more left ventricular hypertrophy and eccentric remodeling than males. Cardiomyocyte cross-sectional area increased in females but not in males. Hormonal status (ovariectomy) had no significant impact on any of the *in vivo* or tissue analysis. We conclude that females developed more left ventricular hypertrophy than males while suffering from severe aortic valve regurgitation and that estrogens did not have any significant protective effects in this animal model. We suggest that heart and body size are significant parameters influencing the degree of LV remodeling in severe AR.

Introduction

Severe aortic valve regurgitation (AR) is a chronic disease that results in progressive left ventricular (LV) dilatation and eccentric hypertrophy. Although not the most frequent valvular disease, it is estimated based on the Framingham study that 13% of the population suffers from some degree of AR (1)]. Mild AR usually will not result in any significant problems. However, it can progress silently for decades and become more severe (2; 3)]. This silent evolution will be accompanied by progressive LV dilatation, hypertrophy and, eventually, by heart failure.

Gender differences in cardiac remodeling, hypertrophy and clinical outcome have been identified in various cardiac diseases such as heart failure, hypertension, aortic valve stenosis and experimental models of pressure and volume overload (4; 5)]. The impact of valvular regurgitation in women (mitral or aortic) has received little attention. Most clinical trials on chronic aortic valve regurgitation have focused mainly on male cohorts and gender specific adaptations of the left ventricle in subjects suffering from chronic severe AR have not been investigated. Therefore, in the present study, we evaluated if left ventricular remodeling and hypertrophy were similar in male and female rats suffering of chronic severe aortic valve regurgitation. The effect of ovariectomy in females was also assessed in order to determine the potential role of estrogens on cardiac remodeling in this valvular disease.

Methods

Animals: 40 female (body weight 225-275g) and 20 male Wistar rats (325-375g) were used in this protocol and studied for a total of 26 weeks. The females were divided in 4 study groups of 8-10 subjects as follows: #1: normal controls (sham-operated: CTRL), #2: chronic AR (AR) and #3: no AR but ovariectomized (CTRL-OVX) animals and #4: chronic AR ovariectomized rats (AR-OVX). Male animals were divided in 2 groups: #1 normal control (sham-operated: CTRL) and #2: chronic AR (AR). This protocol was approved by the Université Laval's animal protection committee and was consistent with the recommendations of the Canadian Council on animal care.

AR induction and echocardiographic longitudinal follow-up.

Severe AR was induced in the animals as previously described by retrograde puncture of the aortic valve leaflets (6)]. Briefly, severe aortic regurgitation was induced by perforating 1 or 2 aortic valve leaflets using a rigid catheter via a retrograde approach from the right internal carotid artery. The procedure was monitored and guided by echocardiography and invasive blood pressure monitoring. Care was taken to induce a similar severity of regurgitation among all groups. AR was considered severe by echocardiography if all the following criteria were met: colour-Doppler ratio of regurgitant jet width (at its origin) to LVOT diameter: >50%; retrograde holo-diastolic flow in proximal descending aorta with end-diastolic velocity >18 cm/s; ratio of time-velocity integral of reversed diastolic flow to forward systolic flow in descending thoracic aorta >60%. These

echocardiographic criteria had to be accompanied by an acute decrease of diastolic blood pressure of >30% when aortic leaflets were perforated. Animals who did not meet the criteria of AR severity during surgery were excluded from the protocol. A complete 2D and Doppler echocardiographic studiy was performed as described elsewhere before and during AR surgery and just before the animals were sacrificed after 26 weeks (7-10)]. Ejection fraction, diastolic function, LV dimensions, relative wall thickness (RWT) and LV mass were all evaluated as described previously (11-14)]. At the end of the protocol hearts were harvested, quickly dissected and freed of connective and adipose tissues. LVs (including the interventricular septum) were weighed as well as the left atrium and right ventricle, snap-frozen in liquid nitrogen and kept at –80^{o}C. A mid LV section was preserved unfrozen, immediately fixed and mounted in paraffin for morphological studies.

Analysis of mRNA accumulation:

cDNA synthesis and RT-PCR analyses were carried out as described elsewhere (15; 16)] using the following amplimer pairs: *glyceraldehyde phosphate dehydrogenase (GAPDH)*, 5'-ATCCCATCACCATCTTCCAG-3' and 5'-CCATCACGCCACAGTTTCC-3'; *collagen type 1 (Col1):* 5'-TGTTCGTGGTTCTCAGGG TAG-3' and 5'-TTGTCGTAGCAGGGTTCTTTC-3'; *Col3:* 5'-CGAGGTAACAGAGGTGAAAGA-3' and 5'-AACCCAGTATTCTCCGCTCTT-3' and fibronectin 5'-GAGAGATCTGGAGGTCAT-3' and 5'-GGGTGACACCTGAGTGAA-3'. Denaturation, annealing and amplification temperatures were 94, 60 (50 for Col3) and 68^{o}C, respectively.

Cardiomyocyte cross-sectional area:

Mid-ventricular sections of the LV fixed in paraffin were stained with Trichrome-Masson. Myocyte cross-sectional area (CSA) was measured as described elsewhere by two blinded observers (17). Sections from 10 animals per group were studied.

Histology: Paraffin-mounted mid-ventricular sections of the LV were stained with Trichrome-Masson. Sub-endocardial views avoiding papillary muscles (3/sections) of the LV wall were digitized and assigned a random number. Analysis of the labeled sections was then done by two observers blinded to group assignation using an image analysis software (Image-Pro Plus, Version 4.5, Media Cybernetics, Silver Springs, MD) in order to quantify the proportion of fibrosis present (blue stain). Results are expressed as mean ± SEM % of blue (fibrosis) staining vs. total staining.

Statistical analysis:

Results are presented as mean ± SEM unless specified otherwise. One-way analysis of variance was performed to compare serial data. Statistical significance was set at a *p* value of 0,05 or less using post-hoc Tukey's test. Data and statistical analysis were performed using GraphPad Prism (version 4.02, GraphPad Software, Inc., San Diego, CA).

Results

Animal characteristics:

AR was well tolerated in all groups. 1 animal died in the male AR group and 1 died in the female AR group. In surviving animals, there was no overt clinical sign of heart failure after 26 weeks. At the end of protocol, ovariectomized (OVX) female rats were heavier than their normal counterparts and males were heavier than all females (OVX or not) from the beginning until the end of the protocol ($p<0,05$, not shown).

Animal hemodynamics (table 1):

All non-AR groups (male or female) had similar systolic, diastolic and pulse pressure (results not shown, p=ns between all groups). As expected, pulse pressure was increased in AR rats and was similar in males and females (not shown, p=ns between all groups). There was no statistically significant difference in systolic, diastolic or pulse pressure between females whether they were ovariectomized or not. There was no statistically significant difference in heart rates between all groups.

Baseline stroke volume and cardiac output (not indexed to body weight) were higher in normal males than normal females. AR severity assessed semi-quantitatively as previously described in the methods section was similar between all groups. AR resulted in a significant increase in stroke volume and cardiac output in all groups. When indexed for body weight, cardiac output (cardiac index)

was however significantly higher in all females (AR or no, OVX or no) compared to males (AR or no). Females with AR had the highest cardiac indexes.

Echocardiographic *in vivo* data after 26 weeks (table 1):

Normal animals (CTRL, no AR):

LV diastolic, systolic diameters and wall thickness were similar between normal control and OVX females. Control males had larger LV dimensions and wall thickness than female hearts (OVX or no). Normal female hearts (OVX or no) were more concentric than normal male hearts as shown by the higher RWT in females. Ejections fractions remained normal and similar in normal females (OVX or no) and males after 6 months.

Animals with aortic regurgitation:

AR resulted in a significant increase in diastolic and systolic diameters in all groups after 26 weeks. LV diastolic and systolic dimensions at the end of the protocol were similar in females (OVX or no) and males. There was no change in LV wall thickness in males whereas there was a trend towards an increase in wall thickness in females with AR (OVX or no). Eccentric remodeling occurred in all groups as shown by the decrease in relative wall thickness (RWT) from baseline, regardless of sex or hormonal status in females. The relative decrease in RWT (ΔRWT) was more prominent in females ($p<0,05$). Ejection fraction decreased significantly in all AR groups compared to their corresponding normal group and

this decrease was not affected by sex or hormonal status (fig. 1, right panel). There were no differences in diastolic parameters as evaluated by pulsed-Doppler of mitral flow (not shown).

Tissue analysis (*in vitro)*:

Macroscopy:

Hearts were collected and weighed at the end of the protocol and results are summarized in figure 1. Results are indexed for the animal's body weight. AR caused a significant increase in indexed LV weight in all groups, male or female, OVX or no (fig 1, left panel). However, the relative increase in LV weight was significantly higher in females (OVX or no) compared to their male counterparts (fig 1, center panel). AR resulted in similar increases in left atrial and right ventricular weight in all groups (not shown, p=ns between groups, male or female, OVX or not) suggesting increased filling pressures. Hormonal status (OVX or no) had not significant effect on the degree of LV, LA and RV hypertrophy in female rats.

Macroscopic LV hypertrophy was corroborated by the analysis of cardiomyocyte cross-sectional area (CSA) as summarized in figure 2. AR resulted in a significant increase in cardiomyocyte CSA in both female groups (OVX or no) whereas there was no significant change in the males.

Sub-endocardial fibrosis was not significantly increased in any of the groups and there were no differences between males and females (OVX or no, results not

shown, p=ns). Hormonal status had no effect on this parameter. We have shown previously in male rats that chronic AR is associated with an increased gene expression of mRNAs encoding for collagen I and III and fibronectin (18; 19)]. In females, similar results were obtained (Figure 3). Collagen I, III and fibronectin mRNA levels were higher in female AR rats compared to sham controls. Hormonal status had no significant effect on this parameter.

Comments:

The present study revealed that female rats with severe AR developed more left ventricular and cardiomyocyte hypertrophy than their male counterparts and that female hearts undergo more eccentric remodeling than male hearts submitted to similar degrees of severe chronic AR. Hormonal status had no significant impact on LV remodeling and hypertrophy in this experimental model.

Significant gender differences have been identified in a wide variety of cardiac diseases. The Framingham Heart Study has shown that normal LV mass in greater in men than in women even after indexing for body surface area [(20)]. LVH is also more prevalent in post-menopausal than pre-menopausal women when compared to age-matched men (21)]. Most investigators interested in studying the gender effect on LVH have focused on pressure overload diseases such as systemic hypertension and aortic valve stenosis (22-24)]. Similarly, most animal models on this topic have been models of pressure overload. In some animal models, estrogen treatment tended to attenuate myocyte hypertrophy [(25-

27)]. Little is known however on gender specific response to aortic valve regurgitation. A protective effect of estrogens on pressure-induced LVH (concentric LVH) does not imply similar effects will be found in predominantly volume-induced LVH models (eccentric LVH) since both forms of hypertrophy respond to different hemodynamics and molecular control pathways.

To our knowledge, our study is the first to assess this question in an animal model of aortic valve regurgitation. Gardner *et al* have evaluated the gender specific response of rat hearts to volume overloading in a model of aorto-caval fistula and found that females fared better than males in their model (28)]. However, this experimental model imposes severe right and left volume overloading on the heart that quickly evolves to overt heart failure: this model, although very relevant as a heart failure model, is not useful for the study of aortic valve regurgitation.

Human data on this topic are as rare as animal data. One retrospective analysis by Rohde *et al* showed no difference in LV remodeling between men and women with aortic regurgitation(29)]. AR severity of the patients included in this study was at the most moderate judging from the reported echocardiographic criteria. Moreover, only 9 women were included in this retrospective study and therefore it is impossible to draw any hard conclusion from this data. This retrospective study did not yield any information regarding the evolution of LV remodeling over time. Klodas *et al* recently reported important findings, on a larger cohort, regarding gender differences in the surgical indications and post-operative outcome of patients undergoing valvular surgery for aortic valve regurgitation (30)]. Their study showed that women and men referred for aortic valve replacement displayed

significant differences in their LV baseline characteristics and post-operative survival. The authors (and corresponding editorial) suggested that echocardiographic criteria unadjusted for women may be inappropriate to determine the optimal timing to perform valve surgery and that surgical correction should be considered earlier in women.

We found that female rats developed more left ventricular hypertrophy and relative eccentricity when exposed to severe AR than males. Estrogens have been shown in other heart disease models to have beneficial hemodynamic and tissue effects to protect against left ventricular hypertrophy, heart failure and ischemia. Estrogens are known to have potent anti-hypertrophic effect in animal models as well as in humans with pressure overload (31-35)]. Considering all of the above, we evaluated if the presence of estrogens could have any significant protective effect in our animals. We found that hormonal status (OVX or no) did not have any significant impact on any of the parameters we measured. On a molecular standpoint, the two types of LVH (concentric and eccentric) respond to pro-hypertrophic stimuli and activating pathways that are significantly different (36; 37)]. It is possible that estrogens are more effective against pressure overload activated pathways then those activated by volume overload. This hypothesis deserves however further investigation and was not the topic of this study.

In our study, systolic, diastolic and pulse pressure were similar in males and females and could not account for the differences found between those groups. The severity of aortic valve regurgitation graded semi-quantitatively by echocardiography was also similar between all groups as well as stroke volume

and cardiac output. However, when cardiac output was normalized for body weight, values were significantly higher in all females. Therefore, for similar degrees of aortic valve regurgitation as evaluated by echocardiography, the relative volume overloading and cardiac workload is much higher in the smaller sized females. This may account for the difference in left ventricular hypertrophy and remodeling we found between female and male rats.

Although results of animal studies are not to be directly transposed to humans, our findings raise some questions regarding the application of similar criteria in males and females to evaluate valvular regurgitation severity. In our animals, similar levels of AR (evaluated by commonly used echocardiographic semi-quantitative criteria) resulted in similar increases in cardiac outputs in males and females but since female hearts were consistently smaller at baseline, relative overloading was proportionally higher in females than in males thus resulting in more remodeling and hypertrophy. Adjustment of LV overloads for body weight and LV dimensions may be appropriate in order to properly assess the true level of overloading. Currently, no such adjustment is recommended.

Study limitations:

As stated earlier, animal data does not necessarily transpose to humans and caution should be used when analyzing this data. The effect of estrogen replacement therapy in ovariectomized rats was not assessed in our study nor was the effect of castration in males. However, since we did not detect any difference between non-ovariectomized, ovariectomized females and normal male rats it is

very unlikely that estrogen replacement therapy would have had any significant impact that was not detected in our study groups. Finally, the evaluation of left ventricular systolic and diastolic function was made non-invasively by echocardiography. No invasive hemodynamic data was obtained. Therefore, it is possible that more sensitive contractility or filling indices measured invasively would have shown differences between the groups. However, the echocardiographic indices we report in this study, even though imperfect, are those usually used in clinical practice.

Conclusions: In this animal model of chronic severe aortic valve regurgitation, females developed more left ventricular remodeling and hypertrophy than males. Hormonal status did not seem to have a significant impact in this model. Our results suggest that total LV overload burden may need to be normalized to body size in order to better appreciate the real degree of overloading that is imposed on the left ventricle in this animal model of chronic severe aortic valve regurgitation.

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Figure legends:

Figure 1: Effect of AR on left ventricular hypertrophy and ejection fraction in females ± ovariectomy vs males after 6 months. Left: absolute LV weight indexed for body mass; Center: relative changes (∆) in indexed LV weight from beginning of protocol; Right: relative changes (∆) ejection fraction from beginning of protocol. Results are expressed as mean ± SEM (n=10/group). *: p<0,05 and **: p<0,01 vs. sham (S) animals.

Figure 2: Cardiomyocyte cross-section area (CSA) after 6 months. Measurement are expressed in arbitrary units (AU) ± SEM (n=10/group). *: p<0,05 vs. sham.

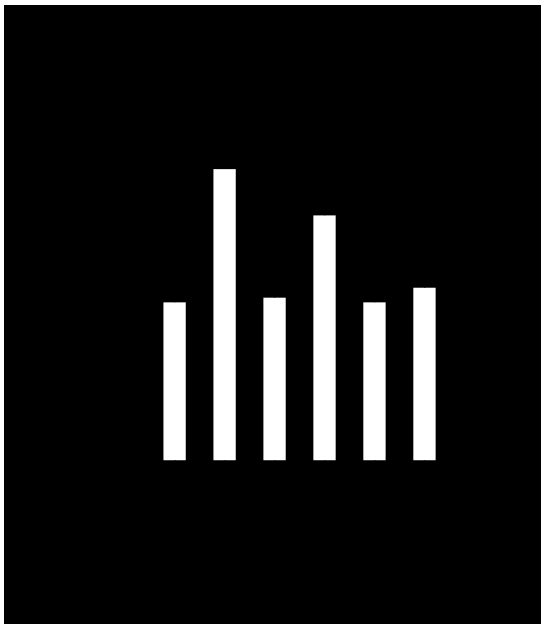
Figure 3: Semi-quantitative evaluation by RT-PCR of collagens type I (left) and type III (center) and fibronectin mRNAs in the LV of female AR rats. Results are expressed as mean ± SEM (n=10/group). *: p<0,05 vs. sham (S) animals.

Table 1. Comparison of echocardiographic and hemodynamic parameters after 6 months

	Female				Male	
	Ctrl	AR	OVX	OVX/AR	Ctrl	AR
EDD, mm	6,8±0,1	10,0±0,4*	6,8±0,2	10,4±0,7*	9,1±0,3#	11,6±0,2*
ESD, mm	4,3±0,1	6,9±0,4*	4,1±0,3	7,0±0,6*	5,3±0,3#	7,9±0,3*
SW, mm	1,5±0,04	1,7±0,08	1,4±0,05	1,9±0,10*	1,8±0,09	1,8±0,04
PW, mm	1,4±0,04	1,6±0,07	1,4±0,11	1,9±0,07*	1,8±0,08	1,8±0,04
RWT	0,43±0,01	0,33±0,01*	0,42±0,02	0,36±0,02*	0,37±0,02	0,31±0,01*
SV, µl	281±20	412±40*	301±10	426±27*	341±10#	423±22*
CO, µl/min	58±3	92±4*	58±4	96±7*	85±3	97±4*#
CI, µl/min/g	0,21±0,01	0,38±0,04*	0,25±0,01	0,32±0,03*	0,13±0,06	0,20±0,01*
EF (%)	61,0±1,2	52,6±3,1*	63,8±3,8	54,4±2,1*	66,1±2,2	53,5±2,0*

Results are expressed as mean ± SEM. Ctrl: sham operated; AR: aortic regurgitation; OVX: ovariectomized; EDD: end-diastolic diameter; ESD: end-systolic diameter; SW: septal wall and PW: posterior wall thicknesses; RWT: relative wall thickness (RWT=(PW+SW)/EDD); SV, CO, CI: stroke volume, cardiac output and cardiac index respectively measured by pulsed-Doppler in left ventricular outflow tract; EF: left ventricular ejection fraction. *: $p<0,05$ vs. corresponding sham-operated animals and #: $p<0,05$ vs. control female rats.

Drolet et al. Fig. 1



Drolet et al. Fig. 2

Drolet et al. Fig. 3.