Effects of the loss of estrogens on the hypertrophic response of the heart to chronic left ventricle volume overload in rats

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Aortic valve regurgitation (AR) can result in heart failure from chronic overloading of the left ventricle (LV). Little is known of the role of estrogens in the LV responses to this condition. The aim of the study aim was to compare LV remodeling in female rats with severe AR in absence of estrogens by ovariectomy (Ovx). In a first study, we investigated over 6 months the development of hypertrophy in 4 groups of female Wistar rats: AR or sham-operated (sham) and Ovx or not. Ovx reduced normal heart growth. As expected, VO from AR resulted in significant LV dilation (42% vs. 32% LV end-diastolic diameter increase in intact and Ovx vs. respective sham group; $p<0.0001$) and CH (74% vs. 70% LV weight increase, respectively; not significant). Increase in stroke volume, cardiac output and loss of systolic function were similar between AR and AR Ovx groups. We then investigated what were the effects of 17beta-estradiol (E2; 0.03 mg/kg/day) treatment on the parameters studied. Ovx had reduced uterus weight in animals by 85% and E2 treatment restored up to 65% of the expected weight. E2 also helped normalize heart size to normal values. On the other hand, it did not influence the hypertrophic response to AR. In fact, it further reduced LV hypertrophy in AR Ovx rats (41% over Sham Ovx + E2). On the other hand, systolic and diastolic functions parameters in AR Ovx + E2 were similar to intact AR animals. Ovx had a significant effect on LV gene expression of several hypertrophy markers. Atrial natriuretic peptide (*Nppa*) gene expression was reduced by Ovx whereas brain natriuretic peptide (*Nppb*) expression was increased. Alpha (*Myh6*) and beta (*Myh7*) myosin heavy chain genes were also significantly modulated by Ovx in sham females. In AR, LV expression of both natriuretic peptide genes were increased as expected. Ovx further increased it for *Nppa* and did the opposite for *Nppb*. Interestingly, AR in Ovx rats had only minimal effects on myosin heavy chain genes whereas they were modulated as expected for AR animals. In summary, loss of estrogens by Ovx in AR rats was not accompanied by a worsening of hypertrophy or cardiac function. Normal cardiac growth was reduced by Ovx but not the response to volume overload from AR. On the other hand,

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ABSTRACT

Aortic valve regurgitation (AR) can result in heart failure from chronic overloading of the left ventricle (LV). Little is known of the role of estrogens in the LV responses to this condition. The aim of the study aim was to compare LV remodeling in female rats with severe AR in absence of estrogens by ovariectomy (Ovx). In a first study, we investigated over 6 months the development of hypertrophy in 4 groups of female Wistar rats: AR or sham-operated (sham) and Ovx or not. Ovx reduced normal heart growth. As expected, VO from AR resulted in significant LV dilation (42% vs. 32% LV end-diastolic diameter increase in intact and Ovx vs. respective sham group; $p<0.0001$) and CH (74% vs. 70% LV weight increase, respectively; not significant). Increase in stroke volume, cardiac output and loss of systolic function were similar between AR and AR Ovx groups. We then investigated what were the effects of 17beta-estradiol (E2; 0.03 mg/kg/day) treatment on the parameters studied. Ovx had reduced uterus weight in animals by 85% and E2 treatment restored up to 65% of the expected weight. E2 also helped normalize heart size to normal values. On the other hand, it did not influence the hypertrophic response to AR. In fact, it further reduced LV hypertrophy in AR Ovx rats (41% over Sham Ovx + E2). On the other hand, systolic and diastolic functions parameters in AR Ovx + E2 were similar to intact AR animals. Ovx had a significant effect on LV gene expression of several hypertrophy markers. Atrial natriuretic peptide (*Nppa*) gene expression was reduced by Ovx whereas brain natriuretic peptide (*Nppb*) expression was increased. Alpha (*Myh6*) and beta (*Myh7*) myosin heavy chain genes were also significantly modulated by Ovx in sham females. In AR, LV expression of both natriuretic peptide genes were increased as expected. Ovx further increased it for *Nppa* and did the opposite for *Nppb*. Interestingly, AR in Ovx rats had only minimal effects on myosin heavy chain genes whereas they were modulated as expected for AR animals. In summary, loss of estrogens by Ovx in AR rats was not accompanied by a worsening of hypertrophy or cardiac function. Normal cardiac growth was reduced by Ovx but not the response to volume overload from AR. On the other hand, Ovx has important effects on LV gene expression and can modulate it in response to 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 in the Western world, it is estimated based on the Framingham study that 13% of the population suffers from some degree of AR.Singh et al. (1999) Secondary moderate to severe AR also occurs in a significant proportion of patients (5-10%) undergoing transcatheter aortic valve replacement (TAVR). Leon et al. (2016) In poorer populations worldwide, mitral or aortic regurgitations are frequent complications of acute rheumatic fever. Rheumatic valve diseases are still occurring at an alarming rate in the third-world and low/middle-income

countries.Zühlke et al. (2017) 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. Blenck et al. (2016)Maric-Bilkan et al. (2016) The impact of valve regurgitation in women (mitral or aortic) has received little attention. Most clinical trials on chronic AR have focused mainly on male cohorts and gender specific adaptations of the LV in subjects suffering from chronic severe AR have not been investigated. Evangelista et al. (2005)Lin et al. (1994)Greenberg et al. (1988) In prior studies, we showed in a rat model of chronic and severe AR that females had a hypertrophic response similar or stronger to the LV volume overload (VO) than males. Drolet et al. (2006) Beaumont et al. (2017) The main difference was that the LV remodeling taking place in females was characterized by increased wall thickening than for males resulting in a relatively preserved morphology (LV walls to chamber diameter ratio). Beaumont et al. (2017) Moreover, AR females showed a tendency for better survival than AR males. More recently, we observed that loss of androgens in male rats was associated with lesser normal cardiac growth and a decreased LV hypertrophic response to severe AR. This suggested a role for this sex hormone in both the control of physiological and pathological cardiac growth or hypertrophy.Beaumont et al. (2019)

It is believed that estrogens can provide a protection against CH development and its main consequence, the evolution towards heart failure (HF). This has been demonstrated in animal models of pressure overload in female rats. Blenck et al. (2016) In the aorto-caval fistula (ACF) rat VO model, males display a faster progression toward HF and poorer survival than females. Dent et al. (2010a) Since sex steroids have a potent effect on differentiation, they could explain a large part of the sex dimorphism observed in cardiac hypertrophy caused by volume overload. Leinwand (2003)

In the present study, we evaluated if LV remodeling and hypertrophy were similarly influenced by estrogens in female rats suffering of chronic severe AR. The effect of ovariectomy (Ovx) in females was assessed in order to determine the potential role of estrogens on cardiac remodeling in this valvular disease. We also wanted to evaluate if Ovx was associated with decreased LV function and potentially worse survival. In a second protocol, we studied the effects of 17beta-estradiol (E2) supplementation in AR Ovx female rats to investigate if it was associated with a reversal of the effects associated with the loss of estrogens.

METHODS

Animals

Fifty-eight female Wistar rats (225 to 250g) (Charles River, Saint-Constant, QC, Canada) were studied. Ovx females were purchased at age 9 weeks one week after ovariectomy. Experimental groups were as followed: Sham-operated (Sham; n=11), Ovx sham-operated (ShOvx; n=10), AR; (n=12) and Ovx AR (AROvx; n=10). AR was induced at age 10 weeks as previously described by perforation of one or two aortic valve leaflets using a catheter via the right carotid and under echocardiographic guidance. Arsenault et al. (2002) Plante et al. (2003) Sham-operated animals had only the ligation of their right carotid. Duration of the protocol was 6 months. Two additional Ovx groups were also studied namely ShOvx + E2 (n=6) and AROvx + E2 (n=8). E2 (17beta-estradiol) was administered using a subcutaneous pellet implanted in the neck liberating 0.03mg/kg/day for 3 months (Innovative research of America, Sarasota, FL). After this period, a second pellet was implanted to complete the protocol. The protocol was approved by the Universite Laval's Animal Protection Committee and followed the recommendations of the Canadian Council on Laboratory Animal Care.

Echocardiography

An echocardiographic exam was performed two weeks after surgery to confirm AR severity and at the end of the protocol 26 weeks later, as previously described. Arsenault et al. (2013) Arsenault et al. (2002) Plante et al. (2003) The regurgitant fraction was estimated by the ratio of the forward systolic flow time–velocity integral (VTI) to the reversed diastolic flow VTI measured by pulsed Doppler in the thoracic descending aorta. At the end of the protocol, the heart and the lungs were harvested and weighed. Heart chambers were dissected, weighted and the LV was then quickly frozen in liquid nitrogen and kept at -80 C until further use.

Gene Expression Analysis by quantative RT-PCR

LV gene expression was quantified for 6 animals per group by quantitative RT-PCR as described elsewhere. Champetier et al. (2009) Pre-optimized primers were from QuantiTect (Qiagen) and IDT (Coralville, Iowa) (Table 1) and SsoAdvanced Universal SYBR Green Supermix (Bio Rad, Hercules, CA) was used. Cyclophilin A was used as the housekeeping gene.

Table 1. Name and symbol of all primer pairs used for gene expression analysis by quantitative RT-PCR. The table also includes catalogue numbers (from IDT or Qiagen) and the size of the amplicon.

mRNA	Symbol	Catalog no.	Amplicon (bp)
procollagen-1 alpha-1	Col1	Rn.PT.58.7562513	134
procollagen-3 alpha-1	Col3	Rn.PT.58.11138874	100
myosin, heavy polypeptide 6, cardiac	Myh6	Rn.PT.58.8646063	150
myosin, heavy polypeptide 7, cardiac	Myh7	Rn.PT.58.34623828	125
natriuretic peptide precursor type A	Nppa	Rn.PT.58.5865224	79
natriuretic peptide precursor type B	Nppb	Rn.PT.58.5595685	108
cyclophilin A	Ppia	Rn.PT.39a,22214830	140
connective tissue growth factor	Ctgf	QT00182021	102
lysyl oxidase, cardiac	Lox	Rn.PT.58.10677971	150
estrogen related receptor, alpha	Erra	Rn.PT.58.5170310	111
estrogen related receptor, gamma	Errg	Rn.PT.58.8028733	141
retinoid X receptor gamma	Rxrg	Rn.PT.58.6519292	103

Statistical analysis

Results are presented as the mean and the standard error of the mean (SEM). Two-way ANOVA analysis was performed and Holm-Sidak's post-test was used for comparison between the groups (Graph Pad Prism 8.1, San Diego, CA). A Student's t-test was used when only two groups were compared. A p-value lower than 0.05 was considered significant.

RESULTS

Effects of overiectomy on the hypertrophic response to chronic volume overload

AR was surgically induced in intact and Ovx Wistar rats at the age of 10 weeks. The protocol had a duration of 26 weeks or 6 months. All animals survived the duration of the protocol. In Table 2 are summarized the characteristics of the animals at the end of the protocol. Sham Ovx females were smaller and their heart lighter. Indexed heart weight for tibial length was lower for sham Ovx compared to intact sham group. When indexed for body weight, no differences were observed. As expected, AR caused important increases in total heart weight as well as for the left ventricle and left atria. This increase was similar for both intact (non-Ovx) and Ovx animals (75% vs. 70%) as illustrated in Figure 1. In order to confirm that Ovx resulted in a loss of sex hormones, we weighed the uterus, a tissue strongly dependant on estrogens. As expected, uterus weight was markedly decreased in both Ovx groups (sham and AR).

As for the animal and heart characteristics described above, most echocardiographic parameters were significantly changed by AR (Table 3). AR severity was similar between both AR groups. LV end-diastolic diameter was smaller in AROvx animals compared to intact AR. This was also the case for the stroke volume and the cardiac output. Fractional shortening (FS; an index of systolic function) was reduced in AR animals. Interestingly, Ovx was also associated with a reduced FS in sham animals. The E wave, representing LV passive filling was significantly higher in intact AR animals compared to Ovx ones.

In Figure 1, we illustrated variations of several parameters mentioned above in AR animals relative to their respective sham-operated group. As expected, AR caused important cardiac hypertrophy in both non-Ovx and Ovx animals compared to sham but this increase in heart weight was similar for both groups. Although a tendency for a greater proportional increase in LV weight and LV end-diastolic diameter caused by AR was recorded for intact animals, this did not reach statistical significance. Fractional shortening (an index of systolic function), LV stroke volume and cardiac output were all modified by AR but again, there was no difference from the hormonal status (Ovx or not).

Table 2. Characteristics of the animals at the end of the protocol. BW: body weight. Values are expressed as the mean +/- SEM. Group comparisons were made using two-way ANOVA analysis and Holm-Sidak's post-test. *: $p<0.001$ vs the respective sham group and §: $p<0.05$ vs non-Ovx group.

Parameters	Sham (n=11)	AR (n=13)	Sham Ovx (n=10)	AR Ovx (n=10)
Body weight, g	428 +/- 15	418 +/- 20	368 +/- 11§	417 +/- 14*
Tibia, mm	51 +/- 0.2	53 +/- 0.3*	50 +/- 0.3§	50 +/- 0.2§
Heart, mg	963 +/- 20	1685 +/- 31*	765 +/- 20§	1304 +/- 25*§
Heart/BW, mg/g	2.3 +/- 0.1	4.1 +/- 0.2*	2.1 +/- 0.1	3.2 +/- 0.1*§
Heart/TL, mg/mm	18.8 +/- 0.3	31.9 +/- 0.6*	15.4 +/- 0.4§	26.0 +/- 0.5*§
Left ventricle, mg	735 +/- 17	1354 +/- 25*	588 +/- 13§	1015 +/- 25*§
Left atria, mg	25 +/- 3	47 +/- 3*	18 +/- 2	32 +/- 2*
Lungs, g	1.7 +/- 0.1	3.2 +/- 0.3*	2.4 +/- 0.2	2.2 +/- 0.3
Uterus, mg	59 +/- 4	59 +/- 3	9 +/- 1§	9 +/- 1§

Table 3. Echocardiographic parameters of sham-operated animals at the end of the protocol. EDD: end-diastolic diameter, ESD: end-systolic diameter, SW: septum wall thickness, RWT: relative wall thickness, FS: fractional shortening, HR: heart rate, bpm: beats per minute, SV: stroke volume, CO: cardiac output and na: non applicable. Values are expressed as the mean +/- SEM. Group comparisons were made using two-way ANOVA analysis and Holm-Sidak's post-test. *:$p < 0.05$ and §: $p<0.05$ vs non-Ovx group.

Parameters	Sham (n=11)	AR (n=13)	Sham Ovx (n=10)	AR Ovx (n=10)
AR severity, %	na	83 +/- 4	na	78 +/- 2
EDD, mm	7.7 +/- 0.1	10.9 +/- 0.2	7.4 +/- 0.1	9.8 +/- 0.2*§
ESD, mm	3.1 +/- 0.1	6.5 +/- 0.3*	3.6 +/- 0.1	6.2 +/- 0.3*
SW, mm	1.1 +/- 0.02	1.4 +/- 0.05*	1.2 +/- 0.04	1.4 +/- 0.04*
PW, mm	1.2 +/- 0.03	1.8 +/- 0.08*	1.4 +/- 0.07	1.5 +/- 0.06*
FS, %	60 +/- 2	41 +/- 2*	52 +/- 3§	38 +/- 2*
RWT, unitless	0.28 +/- 0.005	0.26 +/- 0.011	0.29 +/- 0.010	0.27 +/- 0.007
SV, ml	0.29 +/- 0.01	0.52 +/- 0.04*	0.22 +/- 0.01§	0.40 +/- 0.02*§
HR, bpm	386 +/- 13	379 +/- 9	348 +/- 16	373 +/- 11
CO, ml/min	113 +/- 3	187 +/- 10*	79 +/- 7§	148 +/- 8*§
E wave, cm/s	95 +/- 4	109 +/- 4	83 +/- 4	86 +/- 3§
A wave, cm/s	61 +/- 3	57 +/- 2	59 +/- 7	47 +/- 2
E wave slope	2992 +/- 199	3379 +/- 305	2098 +/- 215§	2850 +/- 135

Effects of E2 treatment on cardiac hypertrophy in AR Ovx females

We then studied the effects of E2 treatment in both Ovx sham and AR rats. As summarized in Table 4, Ovx rats treated with E2 were still smaller than non-Ovx ones (see Table 2 for comparison). On the other hand, indexed heart weight was normalized suggesting that cardiac growth was not slowed in sham Ovx rats receiving E2. AR produced heart and LV hypertrophy and surprisingly, relatively less than in intact and untreated Ovx animals. Uterus weight was increased by E2 treatment to approximately 65% of non-Ovx females.

As for intact and Ovx rats, Ovx females receiving E2 had a similar hypertrophic response to AR. To note, AR was relatively less severe here compared to data from the protocol mentioned above.

As illustrated in Figure 2, E2 treatment partly normalized cardiac growth in Ovx females. Heart and LV weights were significantly increased in Ovx rats receiving E2 compared to those untreated. On the other hand, LV stroke volume and cardiac output were completely normalized by E2 treatment. Systolic function as evaluated by fractional shortening was unchanged be E2 treatment.

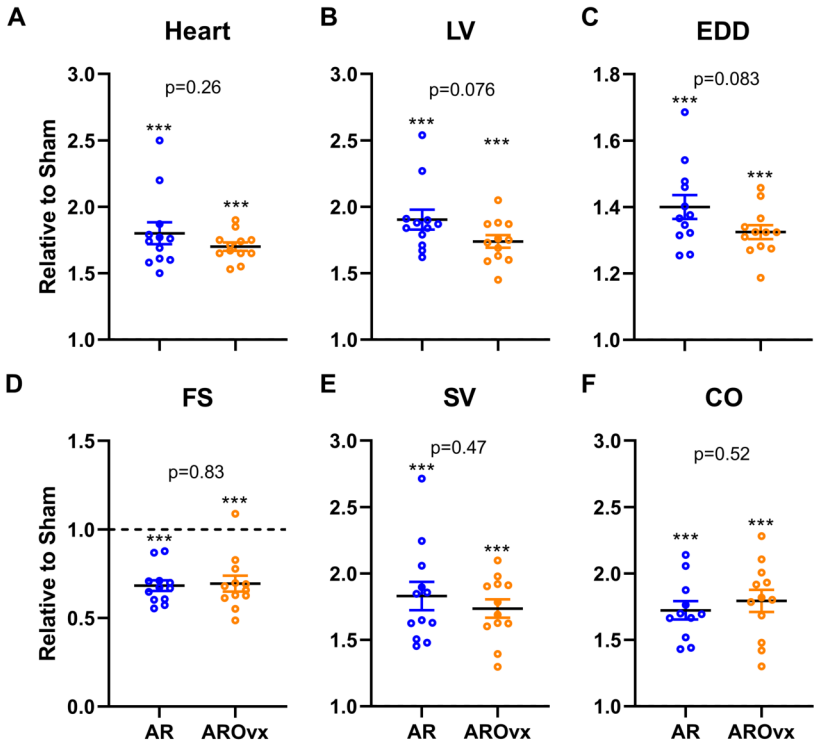

Figure 1. Ovariectomy does not modulate the hypertrophic response triggered by AR. Results are expressed as the ratio of the indicated parameter compared to the mean of the same parameter for the respective sham-operated group. A: Heart, B: LV, Left ventricular weight, C: EDD, end-diastolic diameter, D: FS, fractional shortening, E: SV, stroke volume and F: CO, cardiac output. Results are expressed as the mean +/- SEM. Calculated p values are indicated for comparison between AR and AROvx groups. ***: $p < 0.001$ compared to sham-operated female rats.

LV gene expression modulation by estrogens

We then measured LV gene expression for several hypertrophy markers. *Nppa or Anp* and *Nppb or Bnp* mRNA levels were both modulated by the loss of estrogens in sham Ovx animals (Figure 3A). *Nppa* levels were reduced 60% whereas *Nppb* levels remained stable. Ovx modulated myosin heavy chains gene expression in a similar fashion as the one observed in LV hypertrophy. *Myh6* gene expression was reduced by Ovx whereas *Myh7* was increased compared to intact sham animals. *Nppa* levels were strongly increased in AR animals; this raise being stronger in the AROvx group. We observed the opposite trend for *Nppb* levels, which were more strongly increased in AR females than in AROvx ones. A similar situation was observed for the expression of myosin heavy chain genes in AR rats. *Myh6* gene expression was reduced by AR but less so in Ovx animals whereas *Myh7* was increased by AR but less in Ovx group. Loss of estrogens lead to a decrease in collagen 1 (*Col1a1*) and collagen 3 (*Col3*) gene expression in sham-operated rats (Figure 3B). Collagen 1 and 3 mRNA levels remained normal in AR animals but were slightly more elevated in AROvx animals. The same was observed of mRNA levels of lysyl oxidase 1 (*Lox*) in AR groups. CTGF gene expression levels were unchanged by OVX and were significantly increased by AR. We then tested the expression of genes encoding transcription factors implicated in the control of myocardial energetics (Figure 3C). Estrogen-related receptors (alpha and gamma) and retinoic X receptor gamma mRNA levels were measured in the LV of the animals. ERR alpha levels were reduced by Ovx in sham animals but not further by AR. Moreover, mRNA levels of these three transcription factors were significantly decreased by AR but loss of estrogens restored these levels to normal.

We reported previously that female AR rats kept a relatively normal transcriptional profile for genes related to myocardial energetics. Beaumont et al. (2017) Here, we were interested to see if loss of

Table 4. Animal characteristics of Ovx animals treated with 17beta-estradiol (E2) at the end of the protocol. BW: body weight. Values are expressed as the mean +/- SEM. Group comparisons were made using Student's T-test.

Parameters	ShOvx + E2 (n=6)	AROvx + E2 (n=8)	p-value
Body weight, g	342 +/- 8	320 +/- 11	0.096
Tibial length, mm	48 +/- 0.3	48 +/- 0.3	0.69
Heart, mg	870 +/- 26	1223 +/- 39	<0.0001
Heart/BW, mg/g	2.5 +/- 0.07	3.9 +/- 0.10	<0.0001
Heart/TL, mg/mm	18.0 +/- 0.5	25.6 +/- 0.7	<0.0001
Left ventricle, mg	650 +/- 20	973 +/- 24	<0.0001
Left atria, mg	21 +/- 1	34 +/- 3	<0.0001
Lungs, mg	1.5 +/- 0.1	1.6 +/- 0.1	0.89
Uterus, mg	37 +/- 2.3	40 +/- 3.1	0.55

Table 5. Echocardiographic parameters of of Ovx animals treated with 17beta-estradiol (E2) at the end of the protocol. EDD: end-diastolic diameter, ESD: end-systolic diameter, SW: septum wall thickness, RWT: relative wall thickness, FS: fractional shortening, HR: heart rate, bpm: beats per minute, SV: stroke volume, CO: cardiac output and na: non applicable. Values are expressed as the mean +/- SEM. Group comparisons were made using Student's T-test.

Parameters	ShOvx + E2 (n=6)	AROvx + E2 (n=8)	p-value
AR severity, %	na	66 +/- 2	na
EDD, mm	7.7 +/- 0.1	9.7 +/- 0.1	<0.0001
ESD, mm	3.9 +/- 0.1	5.6 +/- 0.3	<0.0001
SW, mm	1.1 +/- 0.03	1.3 +/- 0.05	0.0019
PW, mm	1.3 +/- 0.02	1.4 +/- 0.10	<0.0001
FS, %	49 +/- 2	43 +/- 2	0.05
RWT, unitless	0.27 +/- 0.007	0.26 +/- 0.010	0.55
SV, ml	0.30 +/- 0.01	0.44 +/- 0.02	<0.0001
HR, bpm	362 +/- 16	351 +/- 10	0.56
CO, ml/min	107 +/- 5	153 +/- 8	0.0005
E wave, cm/s	90 +/- 3	111 +/- 5	0.0024
A wave, cm/s	55 +/- 2	67 +/- 6	0.051
E wave slope	2700 +/- 187	3363 +/- 255	0.0033

estrogens would induce pertubation to this. We thus tested a number of genes related to fatty acids beta-oxidation and glycolysis. In addition, we measured LV mRNA levels of various genes associated to reactive oxygen species (ROS) metabolism. As illustrated in Figure 4, loss of estrogens via ovariectomy had very little effects on LV expression of various genes implicated in myocardial energetics except for one, *Pdk4* (Figure 4A and B). AR reduced the expression of a number of genes namely *Acadvl, Decr1, Hadh, HadhA, Eno3 and Pdk4*. Loss of estrogens did not further modulated those genes in AROvx rats. Among the genes related to ROS metabolism, we observed that NADPH oxidase 4 (*Nox4*) expression was significantly reduced by Ovx. This was reversed by AR. AR up-regulated *Nox2* in both intact and Ovx females.

We then studied if E2 treatment of OVX animals helped restored changes observed in natriuretic peptides and myosin heavy chains gene expression. Interestingly, E2 helped normalize *Nppa* and *Myh7* expression in sham animals (Figure 4). Decreased *Myh6* gene expression in Ovx sham-operated females was not normalized by E2, however. E2 treatment had no effect on gene expression levels in AR animals.

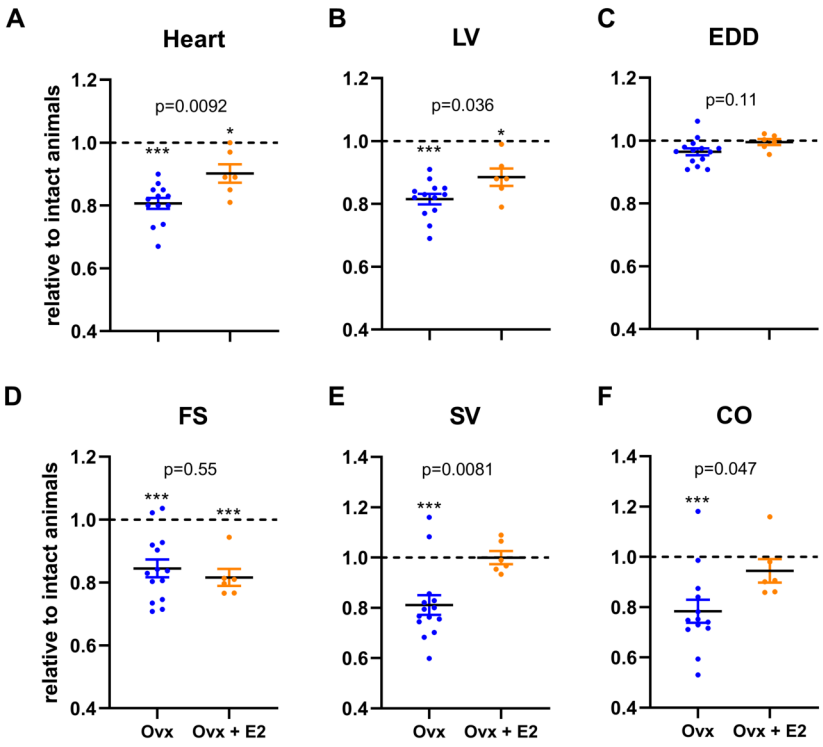

Figure 2. Ovariectomy (Ovx) slows normal heart growth in Wistar female rats and E2 treatment partially restore normal dimensions . Results are expressed as the ratio of the indicated parameter compared to the mean of the same parameter for non-Ovx sham-operated females. A: Heart, B: LV, Left ventricular weight, C: EDD, end-diastolic diameter, D: FS, fractional shortening, E: SV, stroke volume and F: CO, cardiac output. Results are expressed as the mean +/- SEM. Calculated p values are indicated for comparison between Sham Ovx and Sham Ovx + E2 groups. *: $p < 0.05$ and ***: $p < 0.001$ compared to sham female rats.

DISCUSSION

In this study, we observed that loss of estrogens after ovariectomy two weeks before AR induction had relatively little effects on the cardiac response to a LV VO at least at the macroscopic level. Ovx resulted in slower cardiac growth in sham-operated female rats during the 6 months that lasted the protocol. This was be partly reversed by 17beta-estradiol treatment. On the other hand, LV hypertrophy caused by severe VO from AR was similar in all AR groups, Ovx or not and Ovx receiving E2. We had previously shown that LV remodeling from AR in this model involved similar LV dilation in rats of both sexes but relatively more important wall thickening in females. This resulted in AR females compared to sham-operated ones in maintained relative wall thickness (RWT), an index of LV remodeling.Beaumont et al. (2017) RWT also remained stable in sham-operated females after Ovx as well as in all AR groups. Our results suggest that loss of estrogens impacts more strongly normal cardiac growth than the response to chronic and severe VO in the AR rat model.

Study of the roles of estrogens in pathological cardiac hypertrophy has been studies mostly in pressure overload models. It received less attention in volume overload situations such as heart valve regurgitation models or the aortocaval fistula (ACF) model. ACF is a model of global cardiac VO model. It is probably less relevant from a clinical standpoint but remains the most studied VO pre-clinical model. ACF female rats were shown to develop less hypertrophy, to evolve more slowly towards heart failure and to display better overall survival than males. Gardner et al. (2002) This advantage over males was dependant on estrogens as ovariectomy reversed these benefits. Brower et al. (2003) Dent and collaborators characterized this ACF model further and showed that 17beta-estradiol could help normalize the effects of ovariectomy.

Dent et al. (2010b) Some discrepencies seem to exist between findings described in the present study in AR rats and those reported previously in the ACF rat model. A few differences have to be highlighted between these models. In the ACF model studies, evolution towards heart failure was noticed (at least in males) whereas in the AR model, overt heart failure symptoms are a rare occurence and most of the deaths are sudden happening during the active period of the animals at night. Arsenault et al. (2013)Lachance et al. (2009)Plante et al. (2008). Since ACF is a global form of volume overload targeting first the right heart, it is probable than lungs become seriously affected sooner.

More than a decade ago, we had reported that Ovx did not lead to major effects in AR females. Drolet et al. (2006) More recently, we showed that LV dilation caused by AR showed similarities between males and female rats but that the expression profile of many genes related to myocardial energetics was strongly modulated in males but not in females. Beaumont et al. (2017) This suggested that AR females could probably keep a relatively normal myocardial energy metabolism or at least, a better energy substrate use flexibility even in a situation of pathological hypertrophy. In addition, myocardial capillaries density in AR females was not decreased as in males suggesting better oxygen and nutrients availability for surrounding cardiac myocytes. Removing androgens by orchiectomy reversed some of these differences between AR males and females. As observed here, normal cardiac growth in male rats was also dependant on the presence of sex hormones but unlike Ovx females, hypertrophic response to severe VO was clearly decreased in Ocx AR males. Beaumont et al. (2019) Unlike as for androgens, we observed that the estrogens had only minimal effects on the remodeling taking place after the induction of AR. LV dilation was similar just as for the increase in LV stroke volume and cardiac output suggesting that the response to a similar and direct LV stress such as the aortic valve regurgitation induced in our animals required a similar hypertrophic response to accommodate the additional regurgitating blood to pump. Survival was not influence by the hormonal status. This suggest that either estrogens have little effects or that some imprinting of their presence in the early life of the animals still remains.

The steroid hormone 17beta-estradiol is a key player in many biological processes, such as reproduction, development, metabolism, reproduction, cell proliferation and differentiation. Deroo and Korach (2006) Estrogens are implicated in the regulation of many genes and signaling pathways via genomic and non-genomic actions. E2 can bind and mediates its actions via the estrogen receptors (ER) ERalpha and ERbeta, which can then act as transcription factors for specific sets of genes. Membrane-associated receptors such as GPER as well as the other two ERs, can also be activated by E2 resulting in the modulation of cytoplasmic signalling cascades and ultimately regulations of target genes. Murphy (2011) Post-natal heart growth occurs via cardiomyocytes hypertrophy since these cells are post-mitotic. Activation of the ERalpha is required for this heart growth in healthy ovariectomized mice (C57Bl6/J strain) receiving E2 suggesting a central role for this receptor.Karagas et al. (2014) ERbeta does not seem to be involved in normal heart growth in female mice but is believed to be implicated in the protection of the heart during a pathological stress.Mahmoodzadeh and Dworatzek (2019)

Pathological cardiac hypertrophy is associated with an important remodelling of the myocardial structure, a consequence of cardiomyocytes size increase and extracellular matrix rearrangement. Neurohormonal factors as well as mechanic stress cause alterations in myocardial gene expression including the reactivation of the fetal gene programTaegtmeyer et al. (2010). This feature is common to a variety of pathological conditions including ischemia, atrophy, hypoxia, diabetes in addition of hypertrophy. This return to the fetal gene program has long been considered detrimental whereas others have suggested that it protects the heart against irreversible impairment and cell death. Genes associated with the fetal gene program include atrial and brain natriuretic peptide (*Nppa* and *Nppb*), contractile protein beta-myosin heavy chain (beta-MHC or *Myh7*) and early response genes such as *c-myc* and *c-fos* among many others. This reactivation of the fetal gene program in the stressed heart is accompanied with the down-regulation of the adult gene programRajabi et al. (2007). Here, we observed that hypoestrogenism was associated with the modulation of several genes associated with the fetal program in sham females. LV atrial natriuretic peptide (*Nppa*) and contractile protein beta-myosin heavy chain (*Myh6* and *Myh7*) were all significantly modulated by the loss of estrogens. This was also true for other genes often modulated in cardiac hypertrophy such as collagen genes (*Col1a1* and *Col3*), *ERRalpha*, *Pdh4*) and *Nox4*. This modulation was not associated directly with an inhibition or a reactivation of the fetal gene program. For instance, if *Myh6* and *Myh7* genes in the LV of ShamOvx rats followed the usual pattern associated with pathological hypertrophy. On the other hand, *Nppa* was down-regulated and *Nppb* expression remained unchanged. If *Nppa* and *Myh7* gene expression was normalized by E2, surprisingly this was not the case

for *Myh6*.

The results obtained in the present study on female AR rats in conjunction to those we recently published in males demonstrate that sex hormones are not the sole factors intervening in the LV hypertrophic response. Beaumont et al. (2019) Both androgens and estrogens are important for normal cardiac growth. Loss of estrogens by Ovx slows down cardiac growth but E2 treatment helps reverse this effect. Levels of LV hypertrophy are equivalent between AR males and females. Loss of testosterone reduces the extent of LV hypertrophy in AR rats whereas loss of E2 has relatively no effects. In addition, loss of estrogens in AR rats are not associated with a worse transcriptional profile of genes normally regulated in cardiac hypertrophy. In fact, expression of several hypertrophy markers such as myosin heavy chains genes (*Myh6* and *Myh7*) was in part normalized as well as for mRNA levels of *Nppb*, *Err alpha* and *Err gamma*. Again, loss of androgens seemed to provide some benefits to males on this aspect whereas estrogens are mainly neutral in females. Beaumont et al. (2017, 2019) In summary, we did not identify clear negative impact of the loss of estrogens in AR female rats in a chronic setting. Sexual dimorphism in the response to VO seems to rely more on the effects of androgens in males. It is also possible that the effects of sex hormones before gonadectomy are still imprinted later in the life of the animals. Finally, effects of sex chromosomes should not be excluded.

We want to point out several limitations in this study. In the second part of the study where Ovx received E2, AR was less severe (66% in AR Ovx + E2 vs. around 80% for AR and AR Ovx groups) and so was the hypertrophy relative to the respective sham group (41% in AR Ovx + E2 vs. around 70-75% for AR and AR Ovx groups). On the other hand, if one considers the indexed heart weight gain between AR Ovx rats receiving or not E2, the hypertrophic response was similar. The dosage of E2 we used only reversed about two thirds of the expected uterus weight suggesting that it was probably a little low or could not reproduce the natural situation. This could explain in part why cardiac growth was not restored to normal levels. Obviously, continuous release of E2 does not reproduce naturally occurring circadian rhythm of production and release of sex hormones in the body.

In conclusion, we showed that loss of estrogens was not associated with important effects on the myocardial response to severe and chronic aortic valve regurgiation in female Wistar rats.

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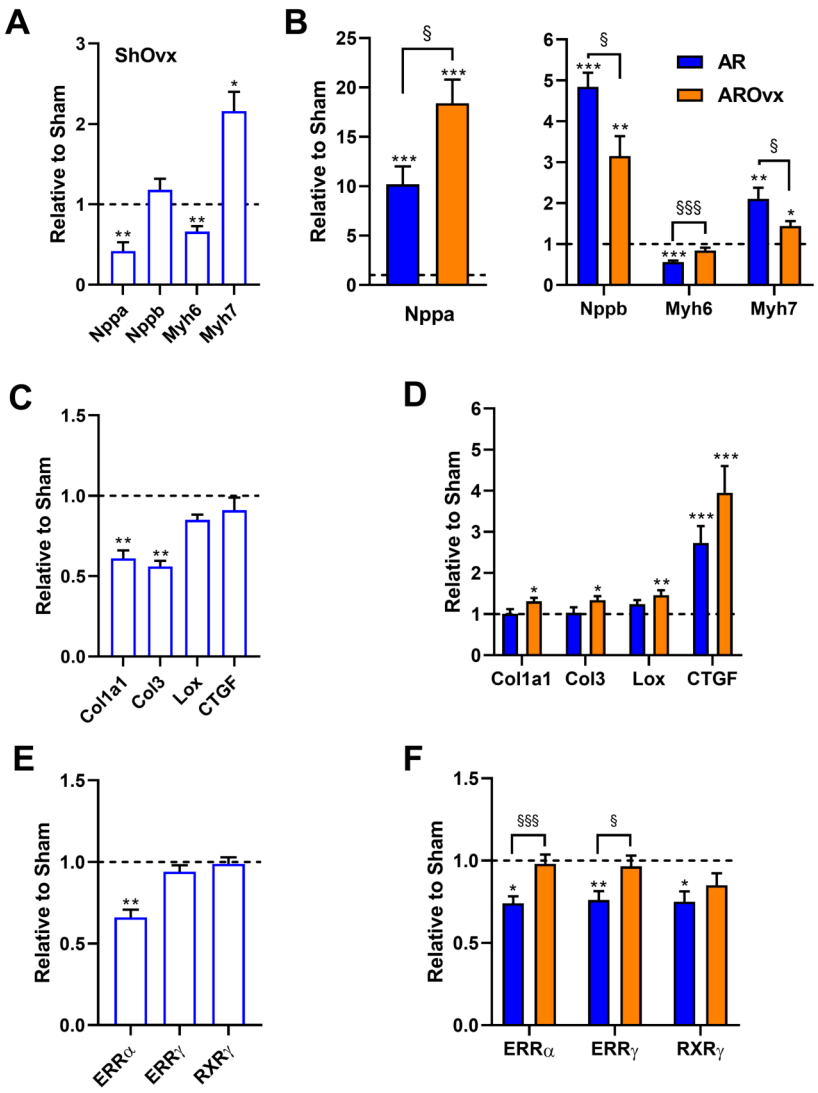

Figure 3. Evaluation by real-time quantitative RT-PCR of LV mRNA levels of genes encoding for hypertrophy markers (A and B), extracellular matrix genes (C and D) and transcription factors implicated in the control of myocardial energetics (E and F). The results are reported as the mean +/- SEM (n=6/gr.) relative to respective sham-operated group. Messenger RNA levels of the respective sham group were normalized to 1 and are represented by the dotted line. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ vs. respective respective sham group. §: $p < 0.05$ and §§§: $p < 0.001$ between indicated groups.

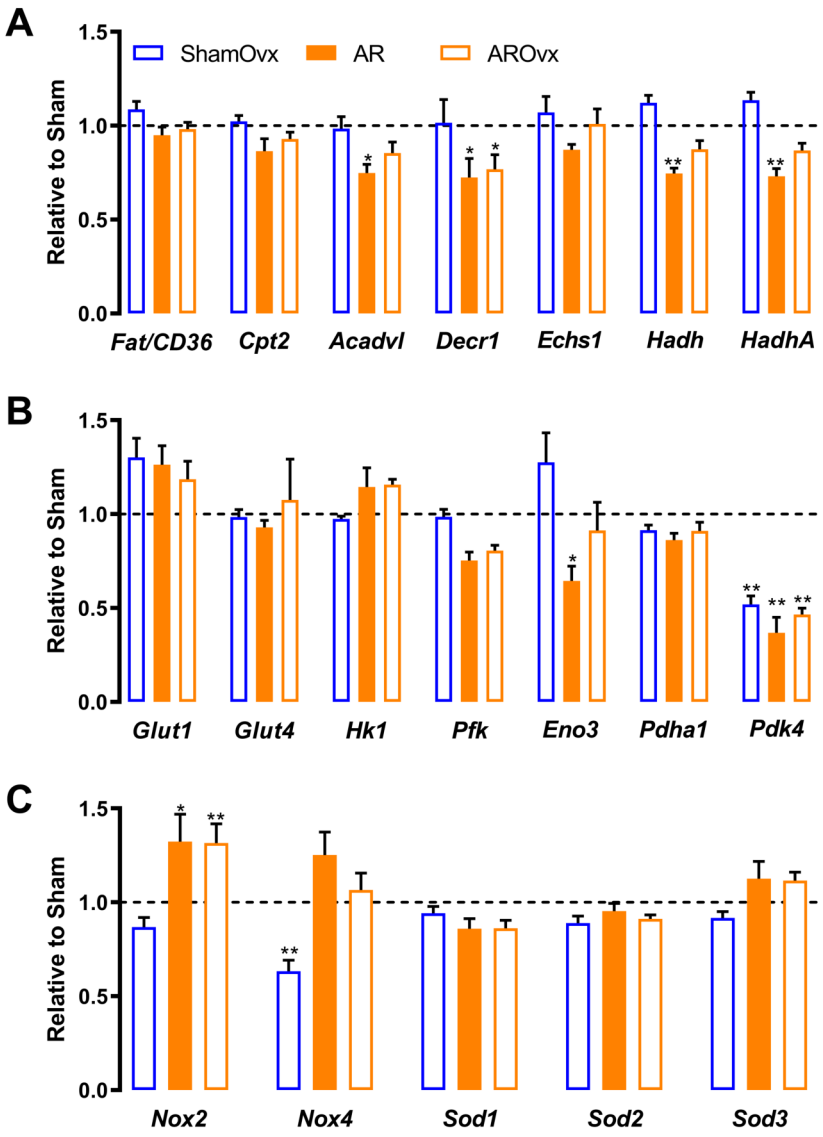

Figure 4. Genes implicated in energy and ROS metabolism are not modulated by the loss of estrogens in sham-operated and AR rats. The results are reported as the mean +/- SEM (n=6/gr.) relative to respective sham-operated group. Messenger RNA levels of the respective sham group were normalized to 1 and are represented by the dotted line. *: $p < 0.05$ and **: $p < 0.01$ vs. intact sham group.

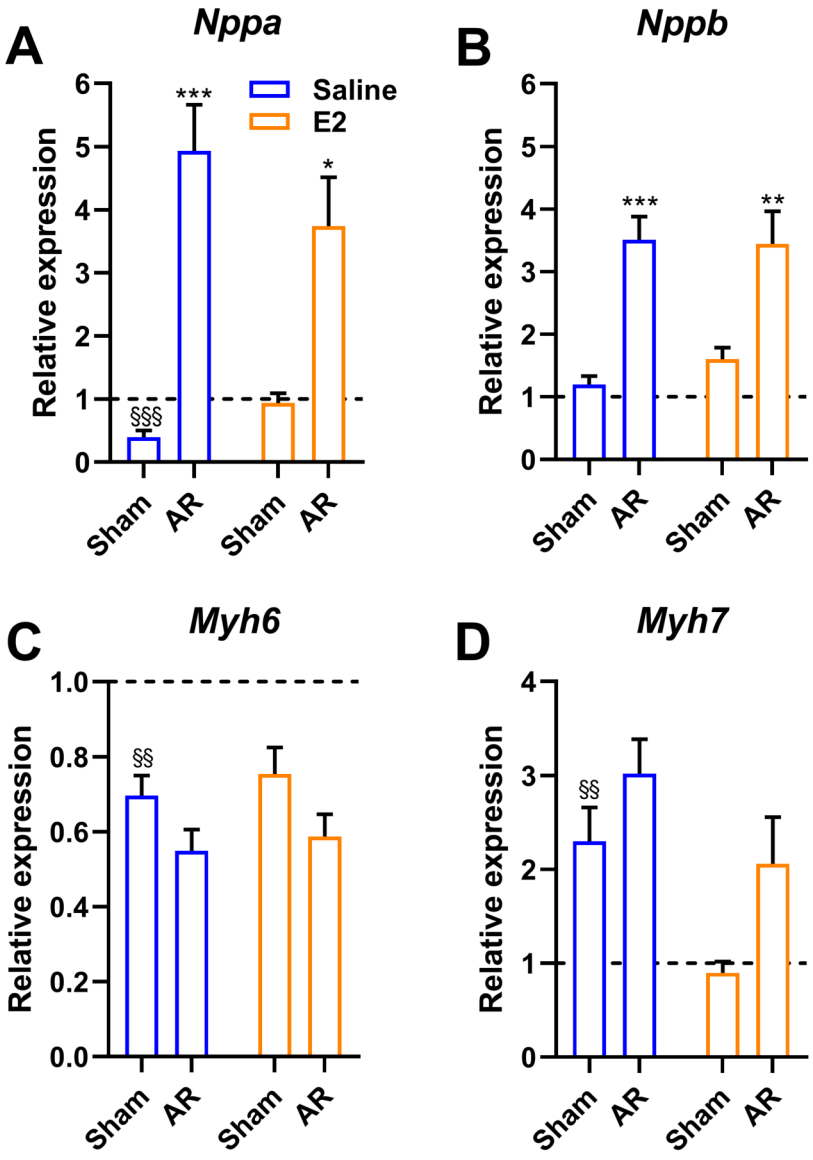

Figure 5. Evaluation by real-time quantitative RT-PCR of LV mRNA levels of genes encoding for several hypertrophy markers in Ovx AR rats receiving or not estradiol replacement. A: Nppa or Anp, B: Nppb or Bnp, C: Myh6 and D: Myh7. The results are reported as the mean +/- SEM (n=6/gr.) relative to respective sham-operated group. Messenger RNA levels of the respective sham group were normalized to 1 and are represented by the dotted line. *: $p < 0.05$ and **: $p < 0.01$ vs. respective intact sham group. §: $p < 0.05$, §§: $p < 0.01$ and §§§: $p < 0.001$ vs. respective non-ovx sham-operated group.