Segmental analysis by speckle-tracking echocardiography of the left ventricle response to isoproterenol in male and female mice

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We studied by conventional and speckle-tracking echocardiography (STE) the response of the left ventricle (LV) to a three-week continuous infusion of isoproterenol (Iso), a non-specific beta-adrenergic receptor agonist in male and female C57Bl6/J mice. Before and after Iso (30 mg/kg/day), we characterized LV morphology and function as well as global and segmental strain. We observed that Iso reduced LV ejection in both male (-8.7%) and female (-14.7%) mice. Several diastolic function parameters were also negatively regulated in males and females such as E/A, E/e', isovolumetric relaxation time. Global longitudinal (GLS) and circumferential (GCS) strains were similarly reduced by Iso in both sexes, GLS by 31% and GCS by about 20%. For the segmental LV analysis, we measured strain, strain rate, reverse strain rate, peak speckle displacement, peak speckle velocity and segmental volume changes in the parasternal long-axis. We observed that radial strain of the LV posterior segments, were more severely modulated by Iso than the anterior wall in males. In females, on the other hand, both posterior and anterior wall segments were negatively impacted by Iso. Longitudinal strain showed similar results to radial strain for both sexes. Strain rate, on the other hand, was only moderately changed by Iso. Reverse strain rate measurements, (an index of diastolic function) showed that similar LV segments to strain were negatively regulated by Iso. Biological sex differences were relatively moderate. Some STE parameters being more severely impacted in one sex compared to the others and vice-versa. Taken together, our study indicates that strain analysis can identify regions of the LV that are more negatively affected by a cardiotoxic agent such as Iso.

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ABSTRACT

We studied by conventional and speckle-tracking echocardiography (STE) the response of the left ventricle (LV) to a three-week continuous infusion of isoproterenol (Iso), a non-specific beta-adrenergic receptor agonist in male and female C57Bl6/J mice. Before and after Iso (30 mg/kg/day), we characterized LV morphology and function as well as global and segmental strain. We observed that Iso reduced LV ejection in both male (-8.7%) and female (-14.7%) mice. Several diastolic function parameters were also negatively regulated in males and females such as E/A, E/E', isovolumetric relaxation time. Global longitudinal (GLS) and circumferential (GCS) strains were similarly reduced by Iso in both sexes, GLS by 31% and GCS by about 20%. For the segmental LV analysis, we measured strain, strain rate, reverse strain rate, peak speckle displacement, peak speckle velocity and segmental volume changes in the parasternal long-axis. We observed that radial strain of the LV posterior segments, were more severely modulated by Iso than the anterior wall in males. In females, on the other hand, both posterior and anterior wall segments were negatively impacted by Iso. Longitudinal strain showed similar results to radial strain for both sexes. Strain rate, on the other hand, was only moderately changed by Iso. Reverse strain rate measurements, (an index of diastolic function) showed that similar LV segments to strain were negatively regulated by Iso. Biological sex differences were relatively moderate. Some STE parameters being more severely impacted in one sex compared to the others and vice-versa. Taken together, our study indicates that strain analysis can identify regions of the LV that are more negatively affected by a cardiotoxic agent such as Iso.

INTRODUCTION

Women represent over lifetime 50% of heart failure (HF) patientsPfeffer et al. (2019). Clinical presentation of HF is different between the sexes. HF is often more ischemic in men, happens at a younger age and leads to reduced ejection fraction (EF). In women, HF happens at older age and often as a consequence of hypertensive disease. Moreover, several cardiomyopathies are more prevalent in women, namely Takotsubo cardiomyopathy. This stress-induced cardiomyopathy (SIC) has been modeled in rodents by creating an acute and severe adrenergic overstimulation using norepinephrine or the non-selective beta-adrenergic agonist, isoproterenol Sachdev et al. (2015).

Beta-adrenergic overstimulation using isoproterenol (Iso) has been often used to induce cardiac toxicity in small rodent models Gomes et al. (2013); Chang et al. (2018); Kudej et al. (1997). Sometimes described as a HF model (systolic and/or diastolic), a cardiac hypertrophy model or a SIC model (Takotsubo-like syndrome)Puhl et al. (2016); Wallner et al. (2016); Sachdeva et al. (2014); Shao et al. (2013a,b); Angelini and Gamero (2019), use of Iso on rats or mice has been the object of an important amount of literature.

Many regimens of Iso administration to rodents have been used in past studies such as a single

high dose bolus, several injections over days as well as continuous infusion using osmotic micro-pumps implanted sub-cutaneously or intra-peritoneally, or micro-pellets at different dosages and for various duration An et al. (2016); Chang et al. (2018); Ali et al. (2019); Shao et al. (2013b); Ma et al. (2011). This diversity of experimental set-ups makes the comparison between studies sometimes difficult as the goals pursued by the authors were often different. In addition, as for many pre-clinical studies, most experiments were conducted in male animals and less often in females, especially in rats. In mice, more studies were performed using females but again, few studies studied in parallel both sexesZhu et al. (2016).

Sex-related differences in the Iso model have been studied using echocardiography in C57Bl6 mice infused for 7 or 14 days (10 mg/kg/day) but relatively few differences in either systolic or diastolic cardiac function were found. Most of the differences were identified at tissue level Zhu et al. (2016). More recently in another study, no sex differences were found in mice treated with Iso for 14 days Grant et al. (2020).

In the present study, we wished to document the left ventricular (LV) response to a beta-adrenergic receptor-mediated insult using the Iso mouse model and to possibly identify morphological and functional sex differences. We used a higher dosage of Iso (30 mg/kg/day) for a longer infusion period (21 days). Using conventional and speckle tracking echocardiography (STE), we evaluated if LV dysfunction was present and then performed a regional study of this dysfunction.

Our results indicate that Iso induced both systolic and diastolic function impairments in mice and that only small sex differences were present in the extent of these. By STE, we showed that the response to Iso is not homogeneously distributed throughout the LV wall as the posterior LV wall seems more sensitive to Iso effects, at least in male mice.

METHODS

Mouse model

The present study was conducted within the Mouse Animal model of Sex Differences and Aging in heart Failure (MASDAF) study, which follows longitudinally C57Bl6/J mice in order to investigate biological sex and aging effects on the LV response to an insult. In this sub-study, we compared the LV response of both males and females (purchased at 8 weeks of age) from Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed 3-5 mice per cage. Nesting material and a shelter was provided. After a week of acclimatization, micro-osmotic pumps (cat. no.: 1004; Alzet, Cupertino, CA, USA), releasing isoproterenol (Iso: 30 mg/kg/day; Sigma-Aldrich, Mississauga, Ont, Canada; n=10 mice/group) or vehicle (saline; Ctrl.; n=8 mice/group), were implanted subcutaneously in the back of the neck and left for 21 days Roussel et al. (2018). The animals were monitored daily by experienced technicians for health and behavior during the protocol. The animals were weighed weekly. No mouse displayed markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress, which would have led to early and immediate euthanasia. Among those, significant loss or gain of weight, grooming and changes in behavior were monitored. The animals used in this study were not euthanized at the end of this period but were let to survive for an additional 12 weeks. Euthanasia was performed under isoflurane anesthesia. The thorax was then opened and cardiac exsanguination performed. The heart was then excised and managed for storage. The protocol was approved by the Université Laval's animal protection committee and followed the recommendations of the Canadian Council on Laboratory Animal Care (2019-360, VRR-19-075).

Echocardiography

Echocardiography (Echo) studies were performed the day before Iso infusion started and 3 weeks later. Echo images were acquired on a Vevo 3100 imaging system (VisualSonics, FujiFilm, Toronto, Canada) by the same investigator and analysed off-line using Vevo LAB software. The investigator was blinded for animal identification but it was not possible to do so for its sex. Animals were anesthetized and positioned on a heated platform ventral side up. The concentration of isoflurane was maintained around 1.5–2.5%, so the heart rate was kept between 400 and 550 beats/minute.

2D echo: M-mode images were recorded to measure diastolic and systolic LV wall thickness from the parasternal long-axis (PSLAX) view and the short-axis (SAX) view at the papillary muscle level. From these measurements, LV Mass was calculated by the VevoLab echo analysis software (VisualSonics) using the following equation: $1.053 \times [(EDD+PW+IVSW)^3 - EDD^3] \times 0.8$ where: EDD is the internal dimension of the LV at the end of diastole, PW is the thickness of the posterior wall at the end of diastole

and IVSW is the thickness of the inter-ventricular septum at the end of diastole. Fractional shortening from M-mode images was calculated using the following equation: (EDD - ESD) / EDD where: ESD is the internal dimension of the LV at the end of systole. Pulsed wave Doppler was used to measure the mitral flow from an apical four-chamber view. Early diastolic peak filling velocity (E wave), peak filling velocity at atrial contraction (A wave), E wave deceleration time and the E/A ratio were obtained. The early-diastolic peak velocity (E'), the late-diastolic peak velocity (A') of mitral valve annulus and E'/A' as well as E/E' were obtained using tissue Doppler. LV volumes, ejection fraction (EF), stroke volume and cardiac output were calculated using the Simpson's rule method from LV chamber area tracings.

Speckle-tracking echo: 2D echo B-mode loops were acquired from the LV PSLAX and analysed using Vevo Strain software (VisualSonics). Images were acquired at 232 frames/s and strain analysis was performed in the radial and longitudinal axes. Three cardiac cycles of the highest quality loops were selected to avoid respiration movements, echo gel artefacts, and obstruction from the ribs. Endocardial and epicardial borders were traced at mid-diastole. LV tracing was started from the anterior wall close from the aorta root to the posterior wall close from the mitral valve. Vevo Strain software then built the dynamic LV tracing for all selected frames. Loops were replayed to confirm validity of border tracking over cardiac cycles and adjustments were made, if needed. LV myocardium was then divided into 6 equal anatomical segments and peak systolic strain calculated for every segment. Strain = (Ls - Ld)/(Ld) where: Ls = Length at end-systole and Ld = Length at end-diastole. From this, the strain rate (SR) was calculated taking into acount cardiac cycle duration. Also calculated by the Vevo Strain software, were the reverse strain rate (a diastolic function index), speckle displacement (mm), velocity (mm/s) as well as segmental volume changes (mm^3). Segmental volume (V) changes were then calculated for each LV segment using maximal Vsystolic - minimal Vdiastolic / maximal Vsystolic (over 3 cardiac cycles) and is expressed in %.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Normality was assessed using the Shapiro-Wilk test. Inter-group comparisons were conducted using Student's T-test using GraphPad Prism 8.4, GraphPad Software Inc., La Jolla, CA, USA). Data from Tables 1 to 3 were analysed using 2-way ANOVA and Holm-Sidak post-test. $P<0.05$ was considered statistically significant. Raw data are provided in the supplemental data section.

RESULTS

Isoproterenol induces systolic and diastolic impairments in male and female mice

Before implantation of the micro-osmotic pump, a complete echocardiography (echo) exam was performed for each animal. Baseline values are listed in Table S1 (Supplemental data). With the exception of those related to the relative size of male and female animals, systolic and diastolic functions baseline parameters were mostly similar between the sexes.

Ten of these eighteen mice of each sex were then treated for three weeks with a continuous infusion of isoproterenol (Iso), a non-specific beta-adrenergic receptor agonist. On day twenty-one, osmotic pumps were removed and the day after, a second echo exam was performed. As depicted in Table 1, Iso treatment had relatively similar effects in male and female mice on M-mode echo measurements. End-systolic LV diameter (ESD) was increased in mice of both sexes resulting in a corresponding decrease in fractional shortening (-7.4% for males and -7.9% for females). As mentioned above, LV volumes were calculated using the Simpson's rule method from PSLAX LV chamber area tracings. From these LV volumes, ejection fraction (EF) was then calculated. Both end-diastolic (EDV) and end-systolic volumes (ESV) were increased after Iso treatment, respectively by 23% and 49% in males and by 14% (not significant; ns) and 60% in females. This resulted in lowered ejection fraction for both male (-8.7%) and female (-14.7%) mice compared to control animals (Ctrl). Stroke volume was maintained in males and tended to decrease in females (ns). Cardiac output was lower in Iso-treated mice.

Diastolic parameters were also modified by Iso. E wave measured by pulse-wave Doppler, was decreased in male mice but not in females as described in Table 2. We then measured E' and A' waves of the mitral valve annulus by tissue Doppler. E' wave was significantly reduced by 34% in males. E/E' ratio, an index of diastolic function was significantly increased by Iso in males (30%). Only a tendency was registered for females (+20%). Isovolumetric relaxation time (IVRT) was significantly longer in both Iso groups compared to controls (+40% in males and +20% in females, respectively).

Table 1. Left ventricle morphology and systolic function in male and female mice receiving or not isoproterenol for 3 weeks. Ctrl: control, Iso: isoproterenol treatment, BW: body weight, LV: left ventricle mass, EDD: end-diastolic LV diameter, ESD: end-systolic diameter, IVS: inter-ventricular septum, PW: posterior wall, RWT: relative wall thickness, FS: fractional shortening, EDV: end-diastolic volume, ESV: end-systolic volume, SV: stroke volume, EF: ejection fraction, HR: heart rate, bpm: beats per minute and CO: cardiac output. Values are expressed as the mean +/- SEM. Two-way ANOVA statistical analysis results are displayed for each factor, sex and iso, respectively. Inter-group p values were calculated using Holm-Sidak post-test. a: $p<0.05$, b: $p<0.01$ and c: $p<0.001$ between Ctrl and Iso groups, respectively. ns: not significant.

Parameters	M Ctrl (n=8)	M Iso (n=10)	F Ctrl (n=8)	F Iso (n=10)	Sex	Iso
BW, g	25.0±0.31	27.9±0.53b	19.9±0.28	20.8±0.45	<0.0001	ns
Tibia, mm	21.0±0.08	21.7±0.10d	20.5±0.08	20.3±0.13	<0.001	ns
M-mode						
EDD, mm	3.8±0.03	3.9±0.07	3.6±0.06	3.6±0.07	<0.001	ns
ESD, mm	2.6±0.08	2.9±0.11a	2.3±0.05	2.6±0.07b	<0.01	<0.001
IVS, mm	0.82±0.02	0.82±0.02	0.76±0.01	0.77±0.02	<0.001	ns
PW, mm	0.84±0.01	0.83±0.02	0.78±0.01	0.75±0.02	<0.0001	ns
RWT	0.44±0.01	0.42±0.01	0.43±0.01	0.42±0.01	ns	ns
FS, %	32.2±1.69	24.8±1.52b	35.4±0.98	27.5±0.86c	<0.05	<0.0001
LV, mg	86±2.1	95±4.1	76±2.6	75±2.7	<0.0001	ns
iLV, mg/g	3.4±0.10	3.4±0.11	3.8±0.14	3.6±0.11	<0.01	ns
Simpson						
EDV, µl	54.6±1.83	66.9±3.62b	44.6±0.74	51.0±2.45	<0.0001	<0.01
ESV, µl	23.4±0.72	34.8±2.78b	17.1±0.34	27.4±2.13b	<0.01	<0.0001
SV, µl	31.2±1.39	32.1±1.91	27.5±0.56	23.6±1.19	<0.001	ns
EF,%	57.1±1.01	48.4±2.19a	61.7±0.55	47.0±2.80c	ns	<0.0001
HR, bpm	494±17.9	415±10.0b	441±16.8	448±9.0	ns	<0.05
CO,ml/min	15.3±0.69	13.3±0.73	12.2±0.56	10.6±0.60	<0.0001	<0.01

Table 2. Left ventricle diastolic parameters in male and female mice receiving or not isoproterenol for 3 weeks. IVRT: isovolumetric relaxation time. Values are expressed as the mean +/- SEM. Two-way ANOVA statistical analysis results are displayed for each factor, sex and iso, respectively. Inter-group p values were calculated using Holm-Sidak post-test. a: $p<0.05$, b: $p<0.01$, c: $p<0.001$ and d: $p<0.0001$ between Ctrl and Iso groups, respectively. ns: not significant.

Parameters	M Ctrl (n=8)	M Iso (n=10)	F Ctrl (n=8)	F Iso (n=10)	Sex	Iso
E, mm/s	662±23.6	551±18,4c	578±15.6	579±14.2	ns	<0.01
A, mm/s	430±21.7	380±9.0	363±10.1	375±16.9	<0.05	ns
E/A	1.55±0.03	1.45±0.03	1.60±0.05	1.57±0.07	ns	ns
E dec.time,ms	20.0±1.20	25.2±1.65	18.8±0.70	22.4±1.88	ns	<0.01
E', mm/s	27.6±1.49	18.3±1.04d	26.1±1.61	22.3±0.81	ns	<0.0001
E/E'	24.2±1.45	31.4±1.57a	22.3±1.42	30.5±2.54	<0.05	<0.001
A', mm/s	19.3±0.44	13.8±0.98c	20.4±1.41	16.4±0.49a	ns	<0.0001
E'/A'	1.43±0.06	1.36±0.04	1.29±0.04	1.43±0.06	ns	ns
IVRT, ms	15.1±0.32	21.1±0.57d	16.1±0.61	19.4±0.93b	ns	<0.0001

In Table 3 is illustrated the evolution of global longitudinal (GLS) and global circumferential (GCS) strains in male and female mice receiving Iso. Global strain measurements take into consideration the entire LV wall comparing the LV inner contour length changes. GLS is calculated from PSLAX view and GCS from SAX view. For both parameters, GLS and GCS, more negative values are associated with better fractional change of the myocardium during the cardiac cycle. In control animals, GLS and GCS values were similar between the sexes. After 3 weeks of Iso infusion, GLS values became significantly

less negative in both males (+31%) and females (+31%). As for GCS, it worsened following Iso similarly in males (+17%) and in females (+23%).

Table 3. Global LV strain parameters in male and female mice treated or not with isoproterenol for 3 weeks. GLS: global longitudinal strain and GCS: global circumferential strain. Values are expressed as the mean +/- SEM. Two-way ANOVA statistical analysis results are displayed for each factor, sex and iso, respectively. Inter-group p values were calculated using Holm-Sidak post-test. c: $p<0.001$ and d: $p<0.0001$ between Ctrl and Iso groups, respectively. ns: not significant.

	M Ctrl (n=8)	M Iso (n=10)	F Ctrl (n=8)	F Iso (n=10)	Sex	Iso
GLS, %	-19.1±0.6	-13.1±0.8d	-20.3±0.8	-14.0±0.9c	ns	<0.0001
GCS, %	-29.4±0.9	-24.3±1.1c	-32.8±1.1	-25.2±0.7d	ns	<0.0001

Segmental analysis using speckle tracking echocardiography (STE) points toward a non-uniform LV response to Iso in males

The LV was divided into six segments for the PSLAX view, as described in Figure 1A. This allowed us to investigate if the effects of Iso infusion were globally distributed along the LV endocardial wall or if one or many segments were more seriously affected than others.

Using segmental analysis, we compared strain (Figure 1) at baseline (Pre-Iso) and after Iso in male and female mice. As illustrated in Figure 2 B-E, radial and longitudinal strains were reduced by Iso in males for all three posterior LV wall segments, whereas anterior ones were left mostly unchanged. Radial strain of two posterior wall segments (base and mid) was reduced in females. In addition, radial strain for all LV anterior wall segments was reduced. Average radial and longitudinal strains (all segments; in black) were negatively modulated by Iso for both male and female mice.

We grouped data from the segmental strain analysis either as from the base, the mid-ventricle or the apex regardless of the posterior or the anterior walls. We did the same for the anterior or the posterior wall data that were analysed together regardless if they originated from the base, mid-ventricle or apex Kudej et al. (1997). As illustrated in Figure 2 E-L, both radial and longitudinal strains were reduced for the base LV segment in male and female mice. Mid LV section radial strain was reduced for both sexes. This was also the case for the longitudinal strain in males as well as the apex longitudinal strain in male and female animals. We then compared if the posterior wall strain was more affected by iso than the anterior wall. In females, both radial and longitudinal strains were reduced by iso relatively to the same extent (-30%). In males, the radial strain was significantly decreased for the posterior wall but not for the anterior one. The longitudinal strain was reduced for both walls but this decrease was more important for the posterior wall than the anterior one (-39% vs. -18%, respectively).

As illustrated in Video S1 (males) and Video S2 (females) (Supplemental data), LV wall deformation after Iso was markedly reduced and sometimes showed features of an infarct in the posterior wall near the apex. When LV movement tendency was expressed using velocity vectors as illustrated in Figure 2, it can be appreciated that both in systole and diastole, the reduction of the length and the changes in the orientation of velocity vectors induced by Iso.

Peak strain rate (SR) represents a systolic function index since it indicates the maximal rate of deformation (strain) during systole. Reverse peak SR happens during the passive LV filling phase of diastole (Figure 3A) and had been proposed as a new index of diastolic function Schnelle et al. (2018).

Changes in radial and longitudinal strain rates (SR) caused by Iso were relatively minor and concentrated on the posterior segments in males and females (Figure 4 B-E). Peak reverse SR showed a pattern reminiscent of those of strain and SR where LV posterior segments were negatively modified by Iso in both males and females (Figure 4 F-H). Several anterior wall segments were also affected but mostly in females.

Peak speckle displacement and peak speckle velocity are analogous to strain and strain rate although they are not expressed relative to a second point in the myocardium as for strain and SR. Original speckle position or speckle velocity is determined from the R-wave and is arbitrarily fixed to 0. Radial speckle displacement and velocity were reduced, mainly for the posterior wall segments in males and females. Displacement was also reduced for the anterior wall in females. In the longitudinal direction, peak speckle

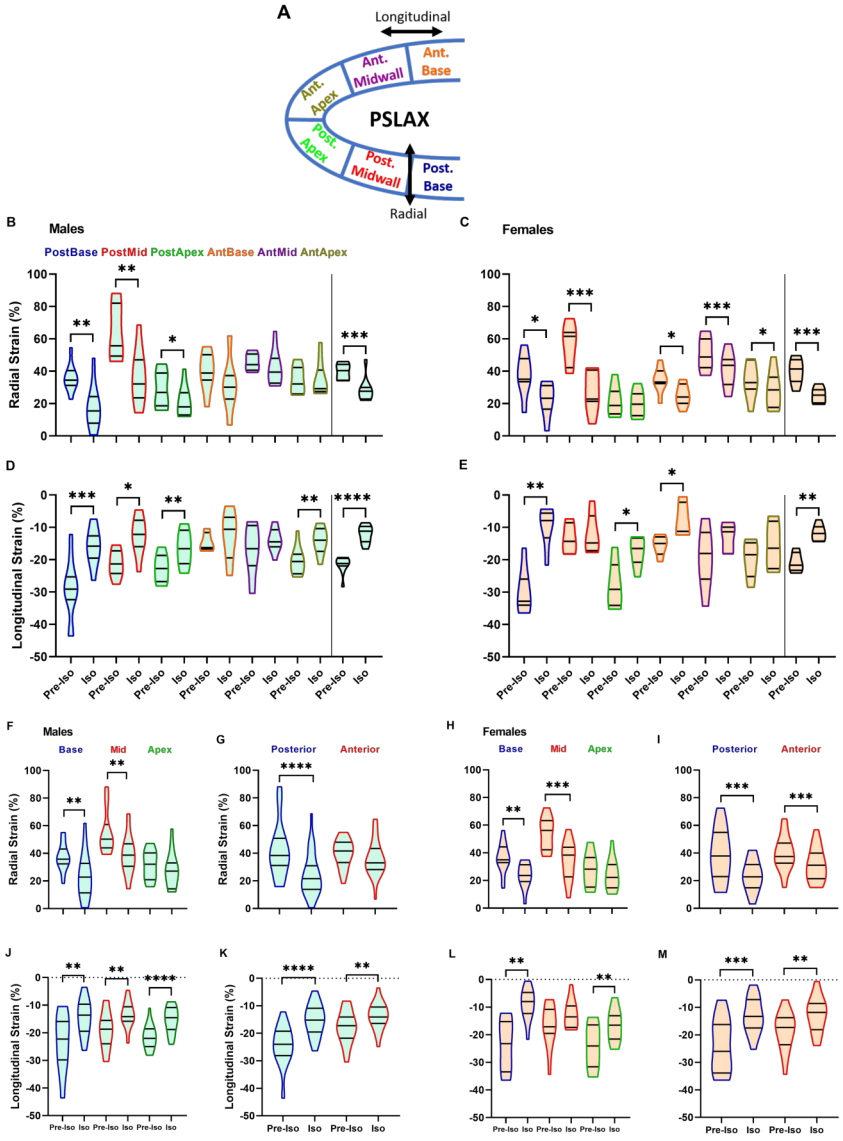

Figure 1. Speckle tracking strain analysis. A. Radial and longitudinal strains can be obtained using the parasternal long axis (PSLAX) view. The six segments are also identified. Characters colors corresponds to those used in the graphs for each of these LV segments. Ant: anterior, Post: posterior. Radial (B-C) and longitudinal (D-E) peak strains were obtained using the parasternal long axis view. The six segments are identified. Characters colors corresponds to those used in the graphs for each of these LV segments. Radial (F-G and H-I) and longitudinal (J-K and L-M) peak strains were grouped either as from the base, the mid-section or the apex or as from the anterior or the posterior segments in males (left panels) and females (right panels). Ant: anterior, Post: posterior. Males are represented on the left panels and females on the right. Results are represented as violin plots (n=8-10). Inner black lines represent quartiles of the data. Significance between groups was calculated with paired Student's T-test. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ and ****: $p<0.0001$ between corresponding pre-Iso and Iso animals.

displacement remained stable in males and was reduced for only one segment (anterior base) in females. A similar situation was observed for longitudinal velocity of the LV wall (Figure S1; supplemental data).

From the endocardial and epicardial combined LV tracings, the Vevo Strain software can produce an estimate of the volume of each LV segment. We determined for each segment both the minimal (diastole) and maximal (systole) volumes and calculated the segmental volume change (in %) as described

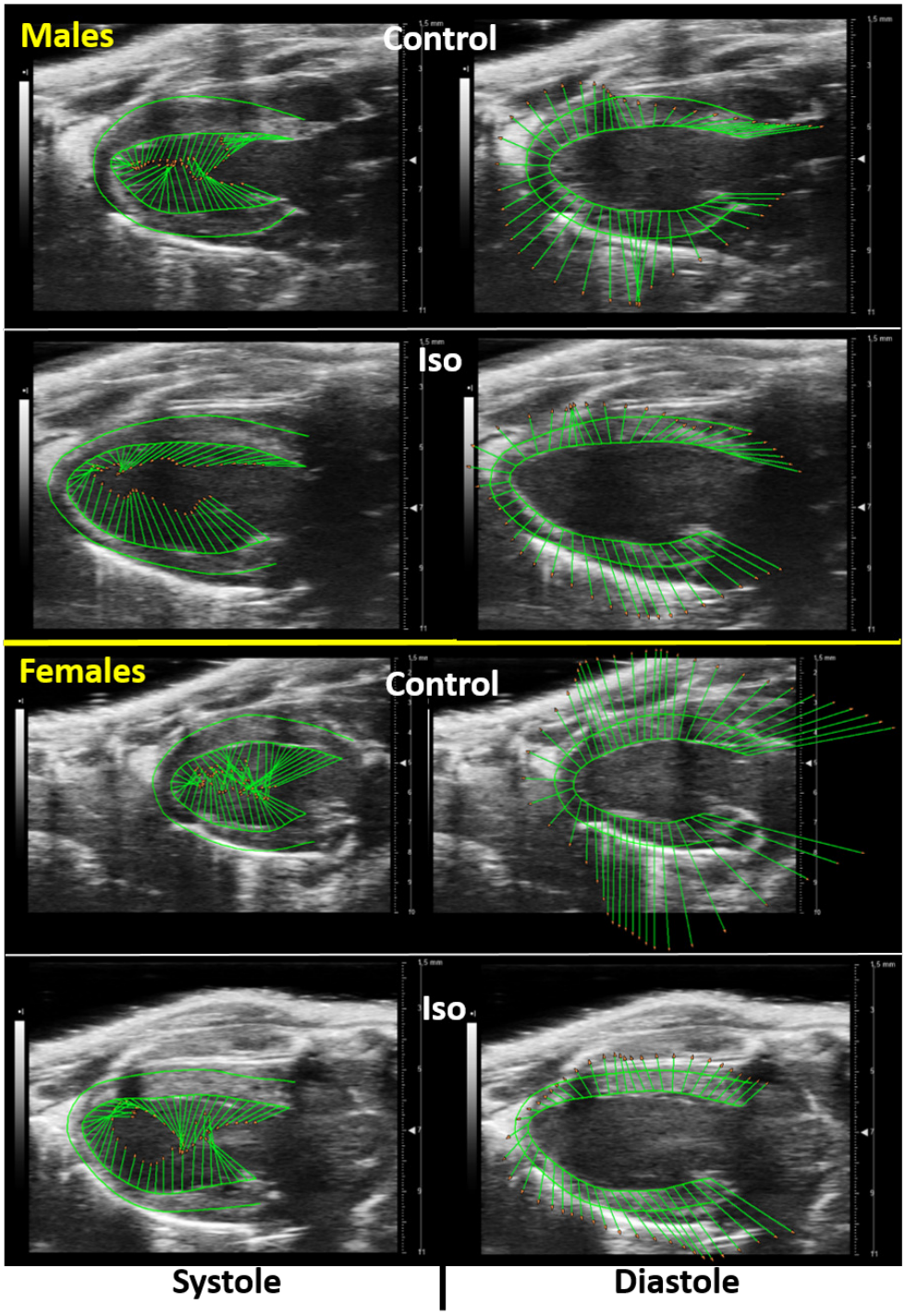

Figure 2. PSLAX LV wall trace tendency before and after Iso. LV wall trace tendency are expressed as velocity vectors for >48 points around the endocardium in systole (left panels) and diastole (right panels) before and after Iso in males (top panels) and females mice (bottom panels). Images of velocity vectors corresponds to the maximal peak (systole) and minimal peak (diastole) of the average curve of all six segments curves for speckle velocity in PSLAX. As evidenced by these PSLAX B-mode views, velocity vectors orientation and length varies during the cardiac cycle. Vertical (radial) and horizontal (longitudinal) components of each vector do not correspond necessarily to the respective peak value of each orthogonal component.

in the Methods section. Results are illustrated in Figure 4. Posterior segments had a general decrease of segmental volume change during the cardiac cycle for both male and female mice. Anterior segments were also reduced in females.

DISCUSSION

In the present study, we demonstrated that the LV response to beta-adrenergic sur-activation using Iso, resulted in both systolic and diastolic function impairments in mice of both sexes. Loss of ejection fraction relative to baseline was more important in females although it resulted in levels similar for both sexes post-Iso. More diastolic function parameters were negatively altered in males compared to females. Using speckle-tracking echocardiography, we proceeded to a thorough investigation of strain, strain rate and other related parameters. In males, we observed that the LV posterior wall was in general more negatively affected by Iso than the anterior wall. This was true for strain in both radial and longitudinal directions. In females, strain in the LV posterior wall was also negatively reduced but the anterior wall was also affected making the effects of Iso more global.

Most baseline values for the different parameters obtained from STE (strain, SR, and rSR) were similar between males and females. Radial peak speckle displacement and velocity for certain segments were significantly smaller in females, which is related to their smaller heart size. To our knowledge, this study is the first to report segmental LV wall displacement and velocity data from normal young adult mice.

Conventional echocardiography

Systolic function as estimated by ejection fraction (EF) was relatively more reduced in females than in males. Baseline EF values were higher in females. Various methods are available for the evaluation of EF by echo. The availability for small animals of four-dimensional (4D) echo LV chamber reconstruction over the cardiac cycle adds another way to estimate EF. In this study, we finally opted for the Simpson's method from LV chamber area tracings in PSLAX instead of 4D echo. Four-dimensional echo, in our hands, probably underestimated LV volume, which lead to incorrect calculations of LV volumes, stroke volume, ejection fraction and so forth. Depending on the method, EF estimates went from being in the 65-70% range using M-mode, 57-62% range using Simpson's method to 55-60% (PSLAX) and 45-50% (SAX) using 4D echo in normal mice. In addition, Iso effects seemed to be masked using 4D echo and variability was higher using this method. See Table S2 in supplemental data section for comparison of the various methods of evaluating EF.

Several factors can limit acquisition of quality 4D echo scans in rodents as described before by several studies Grune et al. (2018); Damen et al. (2017); Rutledge et al. (2020); Grant et al. (2020). One is interference from anatomical structures (sternum, ribs and lungs) that often obscure parts of the heart, making it difficult to visualize and to trace LV walls. Working with those low-quality 4D echo scans can significantly increase intra-observer variability and thus, reduce reproducibility. Therefore, we decided to rely on the Simpson method for volume measurements.

Isoproterenol treatment resulted into a lowering of EF to similar levels in males and females, around 47-48%. It is from the differences in baseline EF between males and females that originated the more important loss in females. Since baseline EF was not significantly different between the sexes using the other methods (M-mode or 4D-echo), we have to remain cautious with the affirmation that Iso treatment had a more negative effect on EF in females. Nevertheless, it is interesting that LV dilatation (EDV variation) was more important after Iso in males, whereas ESV increased more in females suggesting somewhat different paths to systolic function impairments.

Despite these discrepancies between the methods used to evaluate EF, it was clear that Iso increased both systolic LV diameters and volumes in mice. A LV dilation was also apparent in 2D B-mode views, whereas wall thickness seemed to remain remained stable (see Figure 2, Videos S1 and S2). This LV remodeling helped preserve cardiac output although a trend for a decrease was present. It is difficult to affirm that mice were experiencing symptoms of HF after Iso, especially since cardiac output was mostly preserved. HF in small rodents is often recognized by increased lungs weight after euthanasia or decreased exercise performance Gomes et al. (2013). We did not test in our animals resistance to forced exercise.

In the case of diastolic parameters measured either by pulse-wave Doppler or tissue Doppler, many of them were negatively modulated by Iso in male mice. In females, only those measured by tissue Doppler

were affected by Iso and, to a lesser extent than for males. As in humans, defining diastolic dysfunction by echo is difficult although it is clear that Iso treatment caused diastolic function impairments, especially in male mice. Since, the hearts of the animals were not studied *in vitro* afterwards, it is difficult to speculate further for the reason of these observations.

Myocardial interstitial fibrosis is often related to diastolic dysfunction. Iso treatment has been shown to induce fibrosis in mice in many other studies. Collagen production was shown to by more important in male mice receiving Iso than in females Zhu et al. (2016). Among the other mechanisms that have been proposed to explain HF induced by Iso is an increased rate of cardiomyocyte apoptosis Zhuo et al. (2013). Sur-activation of the beta-adrenergic system by Iso also imposes an increased cardiac workload, which is associated with increased consumption of oxygen by the myocardium. This can lead to increased production of reactive oxygen species Ma et al. (2015). An additional mechanism that could explain negative effects of Iso is lipotoxicity. In an acute model of Iso-induced stress cardiomyopathy, rapid lipid accumulation was noticed as soon as two hours after injection. It is not clear if here, in this chronic model, this intracellular lipid accumulation is present or lasts for days but this does exclude a possible toxicity for the cardiac myocytes leading to apoptosis Shao et al. (2013b).

Speckle-tracking echocardiography

In this study, conventional echo was able to highlight LV anomalies both morphological and functional. Our study design did not aim at testing if strain analysis would be more sensitive for early detection of dysfunction as it was done in previous studies An et al. (2016); Li et al. (2014); Peng et al. (2009); Szymczyk et al. (2013). Our goal was to evaluate if additional and valuable information could be obtained using STE such as strain and SR but also reverse SR, speckle displacement, speckle velocity and segmental volume changes.

Using the strain analysis, we observed that the LV posterior wall was in general more negatively modulated by Iso than the anterior wall. The base and the mid-ventricle segments were the most affected and the apex, to a lesser extent. This trend was clearer in males although posterior segments were also impacted in females. Obviously, our Iso treatment being longer and more severe has triggered a compensatory response An et al. (2016). LV dilatation had time to take place mostly in males which has to be accompanied by a concomitant extracellular matrix remodeling. Fibrosis was probably present as described in other studies looking at Iso effects on the mouse myocardium Zhuo et al. (2013); Grant et al. (2020). In addition, this long exposure to Iso most likely brought a general down-regulation of beta-adrenergic receptors leaving non receptor-mediated Iso effects to play a significant part in our observations. The reason for the posterior wall being more sensitive to Iso than the anterior wall still is not clear. Differences in LV regional beta-receptor subtypes density have been reported before. Greater apical beta-adrenergic receptor density or responsiveness has been described in humans, dogs, rats, cats and rabbit hearts Mori et al. (1993); Kawano et al. (2003); Heather et al. (2009); Lathers et al. (1986); Mantravadi et al. (2007). Use of Iso infusion in order to create infarct-like damages and HF as well as for inducing Takotsubo-like syndrome selectively targets the LV apex Paur et al. (2012); Rona (1959); Shao et al. (2013c,b). This basal-apex gradient of beta-adrenergic receptors responsiveness has thus been well-described. Here, our observation made in the PSLAX now includes an additional axis for a possible antero-posterior gradient in beta-adrenegic receptors sensitivity to suractivation. Here, we could only describe this observation without providing satisfactory explanations for the reasons for this. The complex 3D architecture of the myocardium may provide clues but more studies are thus needed to provide new insights about this intriguing observation.

Speckle peak displacement data at baseline show that in the radial direction, posterior segments are more mobile than anterior ones (Figure S1). This is associated with larger radial wall velocities for these posterior LV segments. Here too, it was these posterior segments that were more negatively targeted by Iso for both peak displacement and peak velocity. In the longitudinal direction, baseline values were relatively similar for posterior and anterior segments. Effects of Iso were also less important in the longitudinal direction. Radial displacement and velocity in males were clearly more reduced for the posterior wall. In females, both the posterior and anterior walls were both negatively modulated by Iso.

As illustrated in Figure 2, velocity vectors originating from the LV base (and the apex) show a more important longitudinal component than the radial one. For the mid-ventricle segments, it is the opposite and the radial component of the velocity vectors is more important. Strain and SR measurements are expressed relative to a baseline position at the initiation of systole (EKG R-wave). They do not provide

however, a clear evaluation of the changes in the direction (more radial or more longitudinal) of these vectors after Iso treatment. These vectors also provide information about LV relaxation. This illustrates the high complexity of cardiac contraction and relaxation and the difficulty to assess regional LV dynamic response using only one dimension at a time, here radial vs. longitudinal.

Reverse strain rate has been proposed as an index of diastolic function in mice Schnelle et al. (2018). Considering that most parameters measured by STE are systolic in nature, reverse strain rate can constitute an interesting window to the kinetics of LV relaxation, at least during the passive filling. It is interesting to notice that again, the posterior wall segments in males are the ones with the more reduced reverse SR suggesting LV stiffening caused by Iso seems to target mainly this region.

Segmental volume changes confirmed that the posterior wall was more targeted by Iso in males than its counterpart. In females, the action of Iso was again more global. In the mid-ventricle SAX view, all 6 segments in males had diminished volume changes during the cardiac cycle. This parameter is interesting for its relatively low intra-group variability, which can provide a better sensitivity to the early myocardial response to an insult. Again, our study design did not allow us to test this but this should be investigated in further studies.

STE strain analysis has been performed in the past in Iso-treated mice. In male mice receiving Iso for either 3 or 7 days, global radial and longitudinal strain and strain rate were reduced in PSLAX and only strain rates were reduced in SAX. When concentrating on LV wall segments in PSLAX, the authors found no regional differences suggesting that Iso effects on the LV after 3 or 7 days were mostly global An et al. (2016). Their dosage of Iso used was lower (5 vs. 30 mg/kg here) and the duration shorter making it difficult to make comparison with our work. It is probable that in the present study, more chronic mechanisms took place at the cellular and molecular levels, as mentioned above. Interestingly, after only 3 days, An and collaborators observed increased myocardial fibrosis and hypertrophy. They did not mentioned if chamber dilatation was present but reported LV wall thickening, which was not the case in our mice after three weeks of Iso An et al. (2016).

We thus observe a clear reduction of global strain measurements in our animals and they were similar between males and females. These parameters are highly sensitive to detect cardiac dysfunction but do not provide regional information. Since most sex differences we observed were present at the regional level, global longitudinal and circumferential strains were not informative.

A Takotsubo cardiomyopathy model?

As mentioned above, isoproterenol has been used to develop animal models of Takotsubo-like syndrome. Takotsubo syndrome is defined as a ”transient LV dysfunction (hypokinesia, akinesia, or dyskinesia) presenting as apical, midventricular, basal, or focal ballooning” Napp and Bauersachs (2020). Adrenergic over-stimulation is believed to be an important cause of this cardiomyopathy, which is more frequent in post-menopausal women suggesting possible protective roles for estrogens and/or for the male sex Sachdev et al. (2015).

The rat is usually the preferred animal model to study SIC in animals. It has been studied acutely after a bolus administration of Iso or after a few days of treatment. These short regimens usually allow complete recuperation of systolic function in days or weeks following Iso Shao et al. (2013a); Redfors et al. (2014); Willis et al. (2015); Wallner et al. (2016); Ma et al. (2011). Beta2-adrenergic receptors sarcolemmal localization was proposed for being responsible for the typical apical ballooning associated with Takotsubo cardiomyopathy Wright et al. (2018). Since mid-ventricular and basal forms of this SIC, other mechanisms are involved.

One study has been reported in mice using Iso (one single dose of 400 mg/kg) to induce a Takotsubo-like syndrome Shao et al. (2013b). It did not result in global reduction of systolic function when assessed 2 hours after Iso injection. Segmental fractional wall thickening was measured in SAX view in these mice. Interestingly, two segments had their radial strain severely reduced namely the posterior wall and inferior free wall segments. These segments in SAX are part of the posterior wall in PSLAX. The four other LV segments had an increased strain to compensate in their study Shao et al. (2013b). We did not observe this type of compensation in our mice.

Most attempts to reproduce Takotsubo-like syndrome in rodents so far have relied on acute administration of Iso or catecholamines, suggesting that a acute surge of circulating catecholamines levels or adrenergic over-stimulation are important parts of SIC etiology in patients. Known triggering factors in humans such as the death of a loved one, divorce, financial loss, diagnosis of a serious disease, etc...,

are often related to chronic stress. In fact, circulating levels of catecholamines are seldom elevated in SIC patients Pelliccia et al. (2017). This makes our study possibly relevant to mimic human SIC since our mice were chronically exposed to a beta-receptor agonist. One limitation is that Iso does not stimulate alpha-receptors as catecholamines do, eliminating the vasospasm component of Takotsumo cardiomyopathy Pelliccia et al. (2017).

Study limitations.

Among the limitations of this study, tissue data could not be obtained. The animals were not euthanized at the end of the present study as their longitudinal follow-up was continued for several months. Any tissue data in this context would have been from LVs having a long period of recuperation after Iso treatment. It will be interesting to evaluate how sex hormones and age can influence LV response to chronic Iso in the future. In addition, the study in short-axis (SAX) at the level of the apex, the mid-section and the base the strain and related parameters could be informative in order to correlate changes observed here in PSLAX.

CONCLUSION

Segmental strain analysis in mice can provide information about the regional influence of a toxic cardiac insult such as the continuous infusion with for 21 days. We observed both similarities and sex-related differences in our male and female mice. Both systolic and diastolic functions were negatively modulated by Iso in our animals. Ejection fraction, an index of systolic function was relatively more reduced in females. More diastolic function parameters were negatively changed in males. Differences between sexes were relatively subtle when studied by conventional echo. By STE, we observed Iso had a more global effect on the female LV whereas in males, the posterior wall was more specifically targeted.

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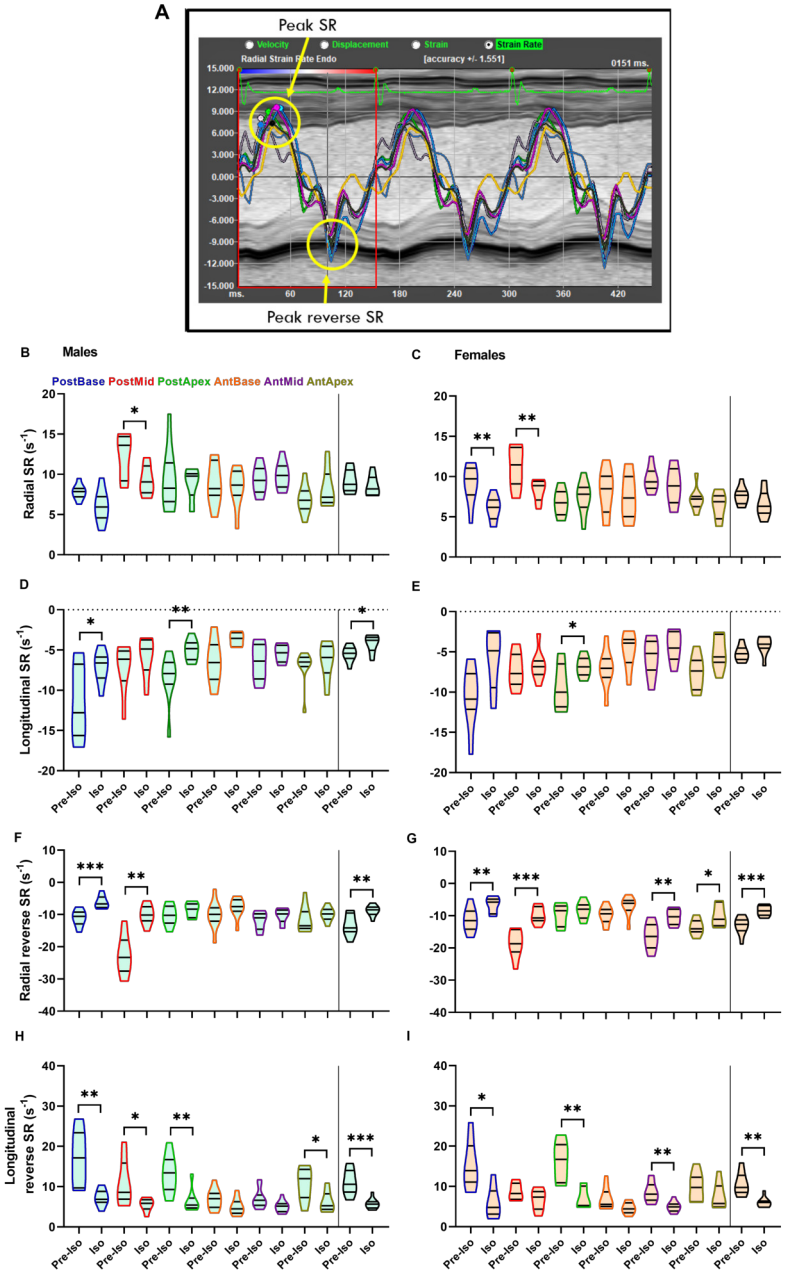

Figure 3. Peak SR and Peak reverse SR (rSR). A. In the background is represented a screen caption of a M-mode loop of three cardiac cycles. In green, the EKG is superposed at the top. Radial strain rate curves are depicted for each SAX segment and as well as an "average" curve in black. Notice that all curves begin at the R wave of the EKG. As evidenced by this representation, the first SR peak (top yellow circle) corresponds to the maximal SR (1/s) whereas the second peak (bottom yellow circle) is inverted and happens during the early stage of LV relaxation as evidenced by the M-mode image underneath. A male mouse after 3 weeks of Iso is represented. B-H. Radial (B-C) and longitudinal (D-E) peak SR as well as rSR (F-H) are illustrated. Characters colors corresponds to those used in the graphs for each of these LV segments. Ant: anterior, Post: posterior, SW: septal wall and FW: free wall. Males are represented on the left panels and females on the right. Results are represented as violin plots (n=8-10). Inner black lines represent quartiles of the data. Significance between groups was calculated with paired Student's T-test. *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$ between corresponding pre-Iso and Iso groups.

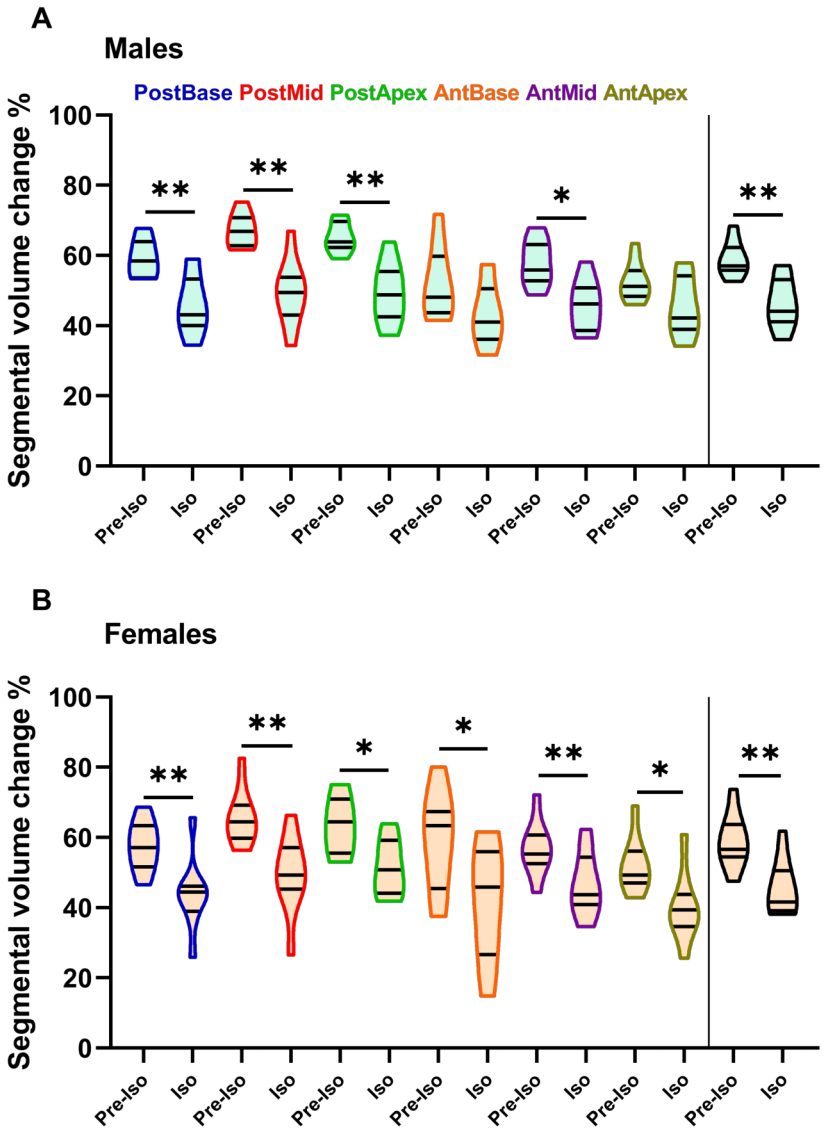

Figure 4. Before-after effects of Iso on segmental volume changes. Relative segmental volume changes are illustrated for males (A) and females (B). Characters colors corresponds to those used in the graphs for each of these LV segments. Ant: anterior, Post: posterior, SW: septal wall and FW: free wall. Males are represented on the left panels and females on the right. Results are represented as violin plots (n=8-10). Inner black lines represent quartiles of the data. Significance between groups was calculated with paired Student's T-test. *: $p<0.05$ and **: $p<0.01$ between corresponding pre-Iso and Iso groups.