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In-situ optical assessment of rat epicardial kinematic parameters reveals frequency-dependent mechanic heterogeneity related to gender

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ABSTRACT

Background

Gender-related cardiac mechanics following the electrical activity has been investigated from basic to clinical research, but results are still controversial. The aim of this work is to study the gender related cardiac mechanics and to focus on its heart rate dependency.

Methods

We employed 12 Sprague Dawley rats (5 males and 7 females) of the same age and, through a novel high resolution artificial vision contactless approach, we evaluated *in-situ* cardiac kinematic. The hearts were paced on the right atria appendage *via* cathodal stimuli at rising frequency.

Results

Kinematic data obtained at rising pacing rates are different between male and female rat hearts: male tended to maintain the same level of cardiac force, energy and contractility, while female responded with an increment of such parameters at increasing heart rate. Female hearts preserved their pattern of contraction and epicardial torsion (vorticity) at rising pacing rates compared to male. Furthermore, we observed a difference in the mechanical restitution: systolic time vs. diastolic time, as an index of cardiac performance, reached higher value in male compared to female hearts.

Conclusion

Our innovative technology was capable to evaluate *in-situ* rat epicardial kinematic at high stimulation frequency, revealing that male preserved kinematic parameters but varying the pattern of contraction/relaxation. On the contrary, female preserved the pattern of contraction/relaxation increasing kinematic parameters.

List of abbreviations

CVD	Cardiovascular diseases
HR	Heart Rate
MHC	Myosin Heavy Chain
Vi.Ki.E	Video Kinematic Evaluation
BCL	Basic Cycle Length
S/D time	Systolic time/Diastolic time
S.E.M	Standard Error of the Mean
PIV	Particle Image Velocimetry
S/D area	systolic area/Diastolic area

1. Introduction

The relationship between protection from cardiovascular diseases (CVDs) and gender has fascinated researchers and cardiologists for decades. The reason is strictly related to the presence of female and male hormones, targeting their effects almost in all part of the cardiomyocyte: ion channel repertoire (Ravens, 2018; Tadros et al., 2014), receptors (Dworatzek et al., 2019), calcium handling machinery (Ayaz et al., 2019), mitochondria (Milerova et al., 2016), intracellular pathways, genome (Kerr et al., 2017) and epigenome (Cong et al., 2013). It emerges that cardiac electrophysiology is, undeniably, affected by estrogen and testosterone (Weerateerangkul et al., 2017), having effects on all phases of the action potential. This would suggest a possible "protection" against the progression towards CVDs for female in premenopausal age compared to male hearts. Nonetheless, all that glitters is not gold. Bazett in 1920 suggested how both sex difference and heart rate (HR) have an impact on the ECG (and

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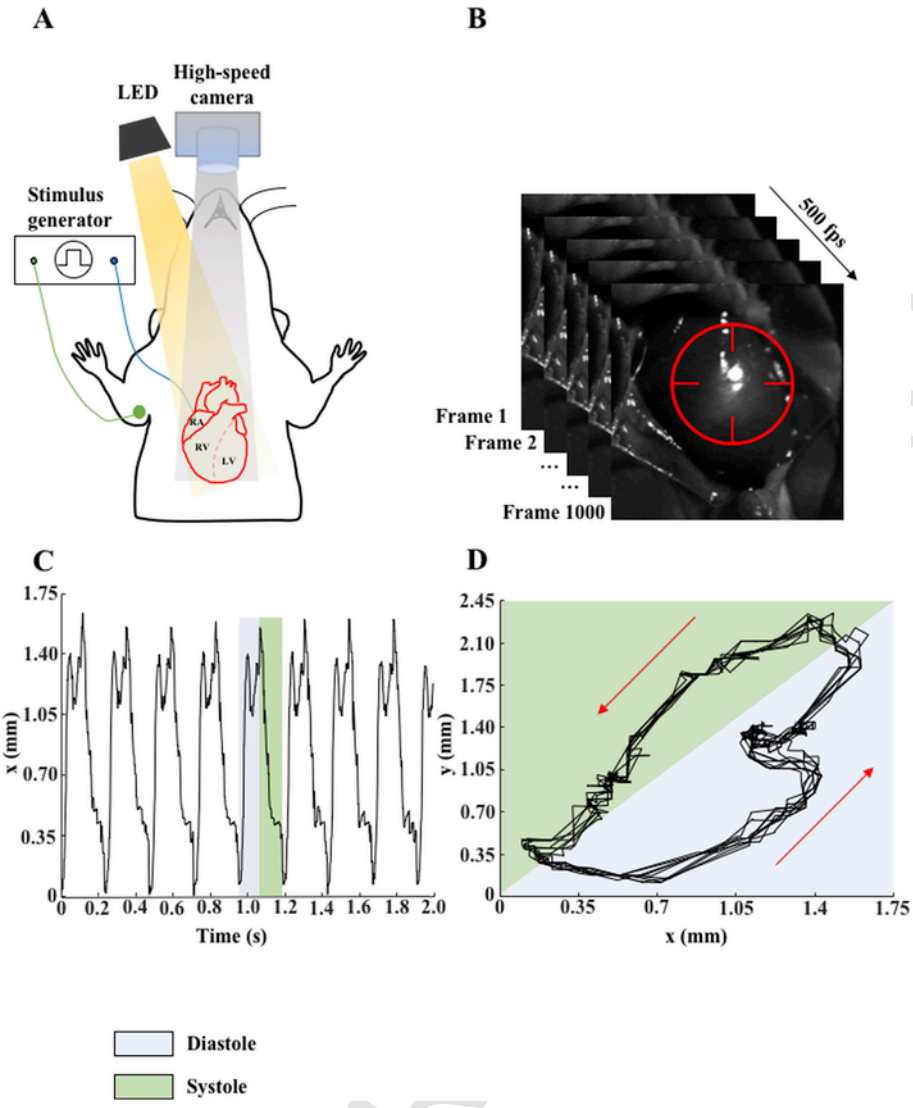

Fig. 1. Schematic representation of the experimental setup. A. The heart (red cartoon) is exposed after median thoracotomy. One end of the stimulating electrode (blue line) is hooked on the Right Atrial (RA) auricle appendage to perform atrial stimulation, while the other end is connected to a stimulus generator (black rectangular box). The current return electrode (green line) is placed on the right forearm. The high-speed camera is placed perpendicular to the heart to record anterior epicardial kinematic. The grey shaded region represents the video camera field of view. The LED is placed beside the camera. The yellow shaded region represents the light beam. RV, Right Ventricle; LV, Left Ventricle; dashed red line, interventricular septum. **B**. Stack of images of rat hearts recorded at 1280 × 1024 pixel at 500 fps. Red target represents the virtual marker positioned in that frame. **C**. Example of the marker movement in x coordinate during 2 s recording. The light blue rectangular indicates the diastolic phase, while the light green rectangle indicates the systolic phase. **D**. Example of trajectories in x-y plane drawn by the marker in 2 s recording. Each loop represents a single cardiac cycle. The light blue and the light green triangles represent the diastolic and systolic phases, respectively. Red arrows indicate the direction of the cardiac cycle. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

he further developed the famous correction QT_c formula that report his name). Women have a long QT in respect to men and this exposes the female at risk of some life-threatening arrhythmias, such as *torsade de pointes* especially when is drug-induced (Makkar et al., 1993). Park et al. (Parks and Howlett, 2013) elegantly summarized the physiological divergences between male and female heart. Moreover, at the single cell level, numerous studies did not observe gender difference in the peak of Ca^{2+} current except Farrell et al. (2010) that demonstrated significant modifications *via* voltage clamp. Cellular contraction has also been taken into account by numerous investigations. Rosenkranz-Weiss et al. reported that female rat ventricular myocytes display high-level of myosin heavy chain (MHC) as well as upregulation of actin mRNA compared to males (Rosenkranz-Weiss et al., 1994). Notably, they found no difference in the α-to β-ratio of MHC proteins, suggesting that those sex differences are abolished after post-translational modifications. Petre et al. (2007) and Schwertz et al. (2004) did not find any gender-related difference in terms of force of contraction in ventricular trabeculae. In detail, Schwertz et al. (2004) found that ATPase activity was higher in female rats at any given extracellular Ca^{2+} concentration compared to male, suggesting that the female contractile machinery had a greater Ca^{2+} sensitivity. Experimentally, the single cell contraction is smaller in female ventricular myocytes compared to male especially when those cells are paced at rapid rates (Grandy and Howlett, 2006). This would indicate that high stimulation frequency may highlight the gender difference in cardiac performance. Some authors showed an increment in isolated ventricular myocyte contraction (Curl et al., 2003), while others reported a reduction (Ren et al., 2003). A robust evidence regarding

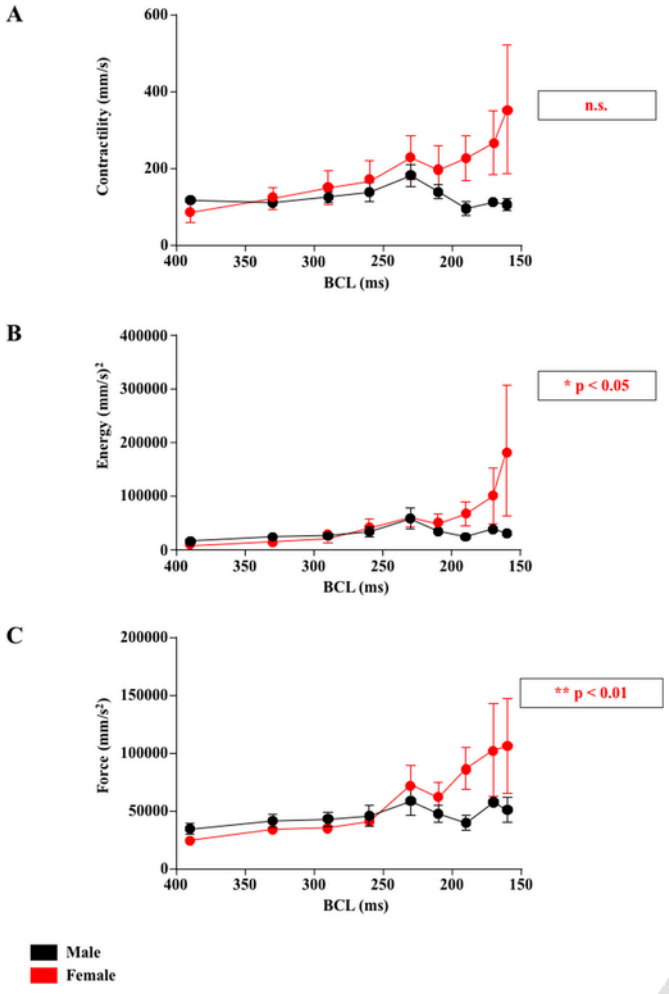

Fig. 2. Effect of the rising frequency of stimulation on epicardial kinematic parameters. Graphs displaying relationship between epicardial kinematic parameters and basic cycle length (BCL) for male (black) and female (red). Trend comparison between male and female for: Contractility (**A**), Energy (**B**) and Force (**C**). Data are shown as Mean ± S.E.M. *p < 0.05, **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the role of female hormone on cardiac activity has been observed in female rats undergoing ovariectomy. To note such differences were completely reversed when 17-β-estradiol was replaced. Androgen receptors are usually present in cardiac tissue and study efforts were mostly focused on their action on intracellular Ca^{2+} handling machinery (Er et al., 2007) rather than contractile performance. Few studies have been performed at multicellular cardiac level after gonadectomy and reported a decrement in the maximum contraction and the peak of Ca^{2+}. Studies on papillary muscle (Schwertz et al., 2004) recapitulated the evidence observed at single cell level, i.e. the greater affinity of female myofibrils to Ca^{2+}, demanding less extracellular Ca^{2+} for evoking the same force of contraction in comparison to male. On the contrary, the same authors reported that in the Langerdorff perfused heart, male generated a greater ventricular pressure compared to female. Such disparate findings indicate that methodological preparations influenced gender dissimilarity.

Based on the observations above, it is evident that rodents are an appropriate animal model for studying the relationship between cardiac performance and gender (Raddino et al., 1989). In particular, rodent models may be well suited for dissecting the biophysical mechanisms underlying the propensity to develop CVDs. In spite of the great efforts and the results obtained at single cell and tissue levels, *in-vivo* investigations on gender-related ventricular kinematic are lacking. We are aware that canonical *in-vivo* gender-related data are merely analyzed from echocardiography (Gori et al., 2014), cardiac MRI and SPECT (Shaw, 2016) exquisitely describing the heart global mechanical function. Unfortunately, these imaging techniques provide only minimal information on kinematic.

In this paper, we adopted the optical-image based technology called Video Kinematic Evaluation (Vi.Ki.E.) (Fassina et al., 2011, 2017; Meraviglia et al., 2016), completely developed in our laboratory, to evaluate *in-situ* high resolution ventricular tissue kinematic in male and female beating hearts, paced at different frequencies.

We observed a frequency-dependent increment of epicardial ventricular contractility, force and kinetic energy in female rat hearts compared to male. Female rats tended to preserve their pattern of contraction and the total perimeter of trajectories at different basic cycle lengths (BCL) resulting in less heart torsion compared to male. Sex difference does not influence the systolic area/diastolic area (S/D area) but it denotes a consistent variation in systolic time/diastolic time (S/D time) between the two groups. Data from our innovative optical-based and contactless technique indicate a profound gender difference in the heart mechanics, opening avenues for further evaluation in the clinical setting.

2. Materials and methods

2.1. Experimental animals

The study population consisted of 12 Sprague Dawley rats (5 males and 7 females) bred in our animal facility (approved protocols: 281/2017, 989/2017). The study was conformed to Italian (D.L.4/3/2014) and European guidelines (2010/63/UE). Rats, 6 months old weighting 250–275 g, were anesthetized with 0.20 mg/kg ip of a mixture of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil; Virbac S.r.l., Carros, France) + 0.15 mg/kg ip medetomidine hydrochloride (Domitor; Orion Corporation, Espoo, Finland). The heart was exposed through a median thoracotomy and suspended in a pericardial cradle under artificial ventilation (RoVent® Small Animal Ventilator, Kent Scientific, CT, USA). Body temperature was maintained constantly at 37 °C with heath lamp radiation and further doses of anesthetic was administered as needed during the experiment. This type of anesthesia invariably suppressed the sympathetic tone in rodents and so our results are not biased by the autonomous nervous system (Tan et al., 2003). Animals where sacrificed with a lethal injection of sodium pentothal as indicated in the protocols.

2.2. In-vivo atrial stimulation

The heart was stimulated with a cathodal suprathreshold train of stimuli (10 Vpp) at different basic cycle length (BCL: 390 ms, 330 ms, 290 ms, 260 ms, 230 ms, 210 ms, 190 ms, 170 ms, 160 ms) by driving the right atrium with a silver electrode (0.05 mm) with one end connected to a stimulus generator (SIU-102, Warner Instruments CT, USA) and the other hooked to the atrial auricle appendage (Fig. 1A). The current return silver electrode has been placed in the right forearm. The stimulus generator was triggered by a function generator (Aim-TTi TG310, RS Components, Milano, IT).

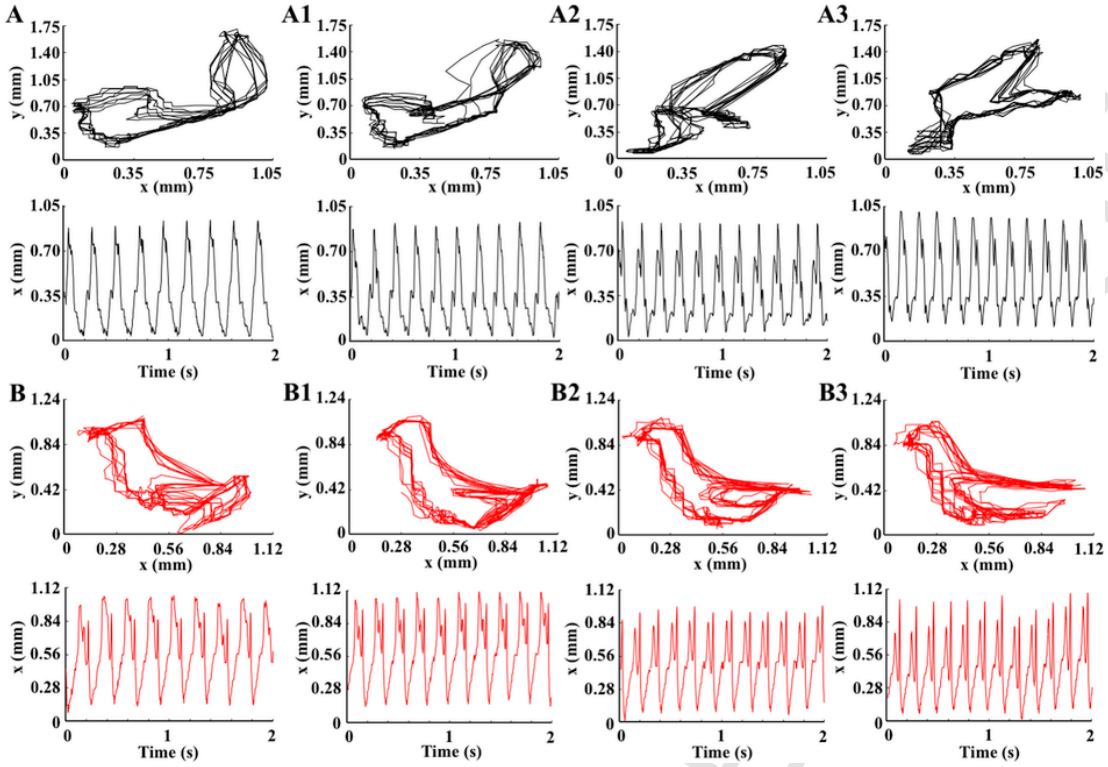

Fig. 3. Contraction patterns at different stimulation frequencies. Examples of male rat heart (black) and female rat heart (red) trajectories and coordinates at four different basic cycle length (BCL). **A, A1, A2, A3**: male trajectories (top panels) and respective coordinates vs. time (bottom panels) at 230 ms, 210 ms, 190 ms and 170 ms respectively. **B**, **B1**, **B2**, **B3**: same as **A**, but for female rat heart. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.3. In-situ optical assessment of kinematic parameters

To evaluate cardiac kinematic parameters, 2 s videos with the temporal resolution of 500 fps (Fig. 1B) were acquired for each BCL, with a spatial resolution of 1280 × 1024 pixel. The camera (Baumer HXC13, Baumer Italia S.r.l., Milano, Italy) was connected with full CameraLink® interface to a frame grabber acquisition board PCIe 1433 (National Instruments, Assago, Italy) and placed at 20 cm above the heart. The camera was equipped with a macro-objective Kowa Industrial Lenses LM35XC, F = 1:2.0, f = 35 mm, picture size 13.8–18.4 mm (RMA Electronics, Hingham, MA, USA). The acquisition board was integrated into a Workstation HP Z220 (Crisel Instruments, Roma, Italy) with 24 GB RAM and the acquisition software was custom made in LabVIEW 2013 Visual Programming Language (National Instruments, Assago, Italy). Only videos with observed 1:1 capture were included in the final analysis.

The videos were elaborated by Video Spot Tracker, an open source software (VST, CISMM, Computer Integrated Systems for Microscopy and Manipulation, UNC Chapel Hill, NC, USA), that enables to place a virtual marker (Fig. 1B, red target) with a chosen kernel on the first video frame. The marker follows the light spots onto the epicardial surface and tracks its coordinates in x and y for each frame. The movement of the light spot is a function of the surface curvature and because it moves back and forth from apex to base during cardiac cycle, it can be assumed as the direct deformation of the epicardial tissue (Supp. Video 1). Afterwards, a custom-made algorithm (Fassina et al., 2017) implemented with Matlab Programming Language (The MathWorks, Inc., Natick, MA, USA) analyzes these coordinates (Fig. 1C), draws the trajectories (Fig. 1D) and returns the following kinematic parameters (Fassina et al., 2017): i) Contractility, expressed in [mm/s], as the average of the maximum module of epicardial velocity for each systolic phase; ii) Force, expressed as [mm/s^2], as the average of cardiac force expended over the entire cardiac cycle during the acquisition period; iii) Energy, expressed as [mm/s]^2, as the average of the cardiac kinetic energy expended over the entire cardiac cycle during the acquisition period. Here we included the novel parameter of trajectory (perimeter, corrected for the number of beats) that measures the marker path length during cardiac cycles. We took advantage of a MathWorks tool named Particle Image Velocimetry (PIV; https://it.mathworks.com/matlabcentral/fileexchange/27659-pivlab-particle-image-velocimetry-piv-tool) as previously used from us (Fassina et al., 2017) and others (Goliasch et al., 2013) for the evaluation of the epicardial torsion. Kinematic data are expressed in the standard SI unit following a conversion: we reconstruct a transformation pixel-to-mm curve by placing the camera at different heights above a graph paper (data not shown).

2.4. Systolic and diastolic area and time

We have extracted from the last complete cardiac cycle of the recorded video the maximal relaxation area (during the diastole) and the minimal contraction area (during systole) for each BCL. Areas were evaluated using a dedicate plug-in from Fiji (ImageJ 9.0, National Institutes of Health, USA) by selecting the frames displaying the maximum relaxation and maximum contraction. Moreover, the systolic and diastolic times were evaluated from the videos as the time interval between the maximum relaxation and maximum contraction and *vice-versa*, respectively.

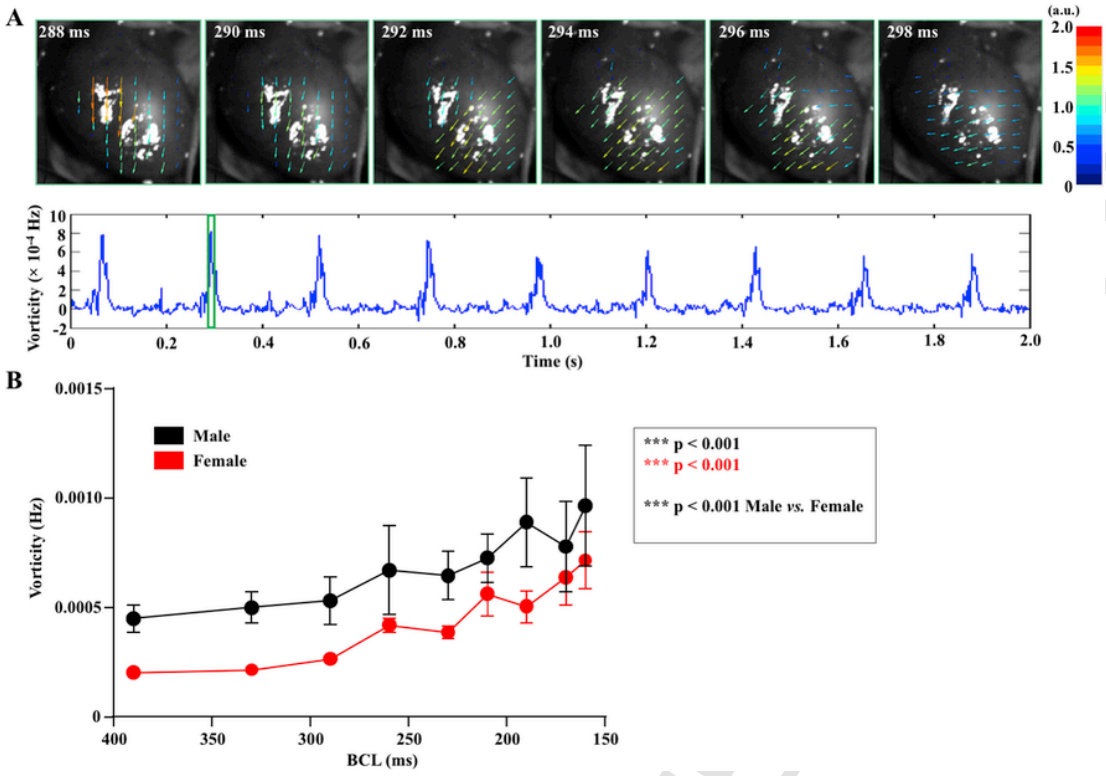

Fig. 4. Epicardial torsion observed by Particle Image Velocimetry. A: Upper panel. Six consecutive frames (step: 2 ms) of Particle Image Velocimetry (PIV) evaluation. The colored arrows represent the velocity vectors; the vector module and colors represent the different epicardial velocities. Bottom panel. Epicardial vorticity parameter (Hz) in 2 s recording. The green rectangle highlights the six frames displayed in top panel. **B**: Relationship between the mean Vorticity parameters and basic cycle length (BCL) in male (black) and female (red) rat hearts. Data are shown as Mean ± S.E.M. ***p < 0.001. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5. Statistical analysis

All data are presented as mean±standard error of the mean (S.E.M.). The normality of the data was assessed with the Kolmogorov-Smirnov test. Kruskal Wallis test and 2-way ANOVA were performed. A value of $p<0.05$ was considered significant. The program GraphPad v.6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analysis and results display.

3. Results

3.1. Frequency-dependent effects on rats epicardial kinematic parameters

We evaluate *via* Vi.Ki.E. technology the global epicardial kinematics at different BCL ranging from 390 ms to 160 ms. We observed that male hearts tend to maintain the same contractility, force and energy (Fig. 2A), while female rat hearts tend to increase the same parameters at the rising stimulation frequency (Fig. 2B). In detail female epicardial force and energy displayed a significant increasing trend ($p=0.0019$ and $p=0.017$ respectively). During our experimental protocol, we noticed that, from BCL = 230 ms onward, an increase of the kinematics parameters occurred in all animals. After this time-point we noticed different trends between male and female. Therefore, we studied in deep the gender-related mechanical behaviour in the following timepoints (BCL: 230 ms, 210 ms, 190 ms, 170 ms) that resemble the range of rat physiological HR. We highlighted this difference in Fig. 3 with representative experiments that display trajectories and coordinates for both male (black) and female (red). As it can be noticed male trajectories (Fig. 3 A-A3 top panels) varied at each BCL indicating different patterns of contraction. On the contrary, female trajectories (Fig. 3 B–B3 top panels) are preserved at each BCL for every cycle. The bottom panels in Fig. 3 A-B display the keeping of 1:1 capture for each cardiac cycle at increasing HR.

3.2. Frequency of epicardial torsion obtained via Particle Image Velocimetry

In order to provide a global information of epicardial torsion following the gender-related pattern of contraction observed in Fig. 3, we sought to evaluate, for each BCL, the cardiac vorticity by analyzing the videos with PIV (Fig. 4A). Fig. 4B shows that cardiac vorticity parameters display an increasing trend statistically significant for both gender during the stimulation protocol ($p=0.0006$). Moreover, the male trend was statistically significant compared to female trend ($p<0.0001$).

3.3. Geometrical spatiotemporal variation at different BCL

The dissimilarity of contraction patterns in male rats at rising frequency suggested that the frequency-dependent epicardial deformation can be related to gender. We sought to investigate the mechanical behaviour at different BCL for both trajectory's perimeter and the maximal contraction (systolic) and maximal relaxation (diastolic) areas. As expected, the data displayed a not-preserved perimeters (Fig. 5A) (ranging from 5.890 ± 0.5597 mm to 3.747 ± 0.3422 mm) over different BCL in male rat hearts (black) compared to female (red) (rang-

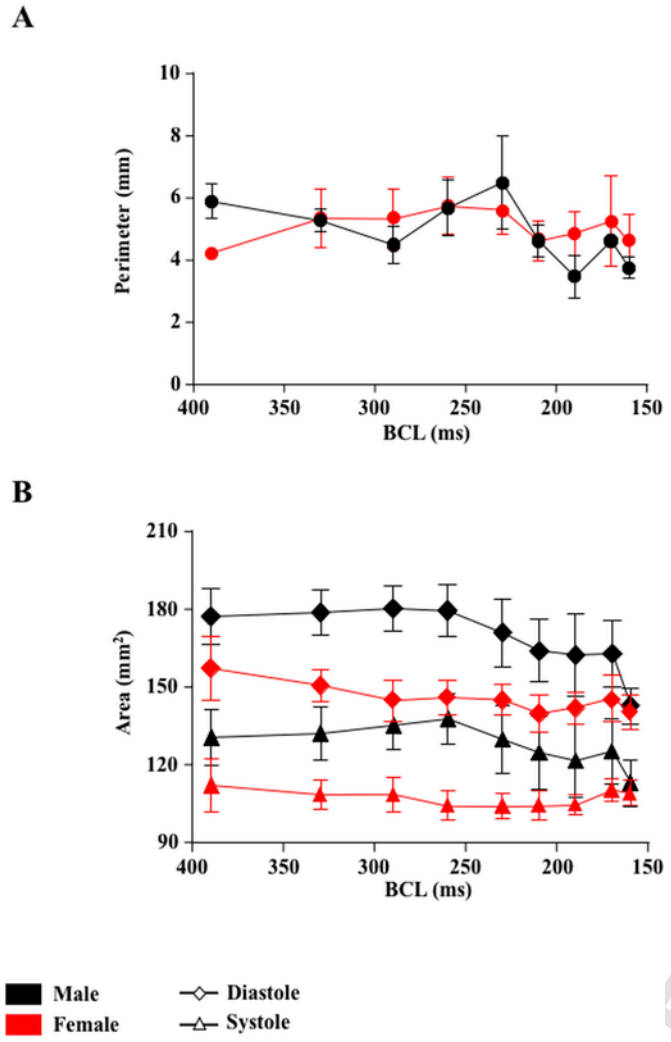

Fig. 5. Relationship between perimeter and area *vs* basic cycle length. A: Male (black) and female (red) perimeter corrected for the number of acquired cardiac cycles for each basic cycle length (BCL). **B**: Average systolic and diastolic area for each BCL in both male (black) and female (red). Diamond: diastole; Triangle: systole. Data are shown as Mean ± S.E.M. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ing from 4.220 ± 0.1566 mm to 4.566 ± 0.8934 mm). The male epicardial areas (Fig. 5B) during maximal systolic and diastolic phases were higher compared to female rat hearts (by ca. 11%); however male areas were more statistically spread at higher frequency in contrast to female, suggesting once more a preservation of the mechanical phenotype of cardiac beating over different BCL in female rats.

3.4. Rising stimulation frequency unmasks gender difference at the single cardiac cycle

We thus recognized that sex differences can be also investigated during the single cardiac cycle extracted from different BCL. Therefore, we plotted the S/D time for both male and female rats beating hearts at rising frequencies of stimulation (Fig. 6). We demonstrated that while S/D area was preserved for both male and female hearts (average male 0.737 ± 0.026, average female 0.753 ± 0.029), paradoxically the S/D time differed (Fig. 6). In the male rats we observed a significant increasing trend (from 0.359 ± 0.029 at 390 ms to 0.913 ± 0.049 at 160 ms, $p < 0.0001$). A significant increasing trend has

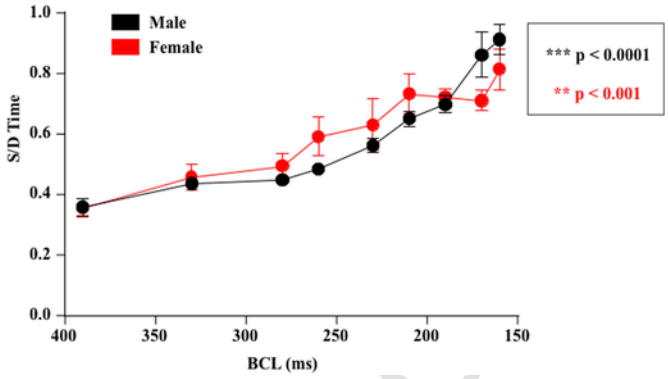

Fig. 6. Mechanical restitution of systolic/diastolic time. Simultaneous plot of systolic/diastolic time (S/D time) in both male (black) and female (red) rat hearts. Data are shown as Mean ± S.E.M. **p < 0.001, ***p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

also been observed for the female rat S/D time (from 0.357 ± 0.031 at 390 ms to 0.815 ± 0.067 at 160 ms, $p < 0.0003$). The S/D time data suggest a different gender-related mechanical restitution at high pacing rates.

4. Discussion

In this study, we applied our high spatial and temporal resolution video kinematic technology to understand the gender-related response of epicardial mechanics during atrial pacing. An additional goal was to examine whether female propensity to cardiac protection against CVDs can be unraveled thanks to our optical-based experimental protocol. From a physiological standpoint it is well known that female has low BMI, low LVED pressure (Wingate, 1997) but higher velocity of contraction suggesting that women are in constant hyperdynamic state (Buonanno et al., 1982). In clinical research, sex difference in cardiac performance is unmasked during physiological exercise (Bombardini et al., 2008) and with RA pacing during cardiac catheterization (Wainstein et al., 2012). At the pathological level, gender has an influence on the manifestation of myocardial infarction (Mehta et al., 2016), with the incidence of ischemic heart disease being much lower in premenopausal women than age-matched men (Mehta et al., 2015). However, the incidence of CVDs after menopause exceed that of men (Blenck et al., 2016). Only recently, sex differences have been considered in clinical and preclinical studies for CVDs (Consideration of Sex as a Biological Variable in NIH-funded Research, 2015). It is also known that sex differences in ventricular mechanics occur during acute physiological challenges, exacerbated by adrenergic stimulation (Williams et al., 2017). In this work, we showed that *in-situ* female beating hearts tended to show a "kinetic reservoir" due to a possible female hyperdynamic state (Buonanno et al., 1982) in terms of contractility, energy and force that can be expended at lower BCL in accordance with the positive role exerted by estrogens on the excitation-contraction machinery (Ravens, 2018). However, we cannot exclude the gender-related difference in the excitation-contraction (EC) coupling machinery including the SERCa activity (Bupha-Intr et al., 2009; Farrell et al., 2010). In agreement to Williams et al. we would expect, for female hearts, an acute ventricular mechanical adaptation to the rising pacing rate. We also notice that female rats data dispersion occurred at high frequency of stimulation according to what has been measured in human females during exercise by Bombardini et al. Moreover, our video kinematic technology captured a change in the mechanical pattern of

epicardial mechanics (trajectories) for male hearts starting from BCL = 230 ms, whereas female hearts trajectories seem to be unaltered at rising frequency of stimulation. This is further confirmed by PIV and trajectory perimeter analysis where the epicardial vorticity and perimeter variation were higher in male compared to female. Because our technique can be applied during open chest surgery (Fassina et al., 2017) we investigated whether such changes may impact on the area of contraction of maximal ejection (systolic phase) and on the area of relaxation of maximal ventricular filling (diastolic phase). Epicardial area changes during heart stimulation and it is an index of ventricular ejection and filling (McCulloch et al., 1989), reflecting the Frank-Starling mechanism which leads to inotropic effect. Accordingly, the systolic and diastolic areas (bigger for male in respect to female, as expected) tended to decrease in male at increasing frequency of stimulation, whereas they seem to be preserved in female. This would suggest that the acute cardiac mechanical adaptation observed in male is necessary to compensate the nonappearance of the "kinetic reservoir" hypothesized in female rat hearts.

One of the echocardiographic index used in clinics is the ratio between systolic and diastolic time intervals (Sarnari et al., 2009). This parameter, together with the rising frequency of stimulation, can provide the mechanical restitution of the heart (Franz et al., 1983). Vi.Ki.E. can be adopted to measure S/D time at high temporal resolution extracted from our coordinates in x and in y. We observed a rising S/D time ratio in both male and female hearts displaying the difference in mechanical restitution. Moreover, the tendency to reach important *quasi*-pathological values for S/D time ratio, i.e. > 1.0 has been observed only in male rats, which is in accordance to the major masculine CVDs-susceptibility observed in humans.

5. Conclusions and limitations

Our video technology unmasked gender-related kinematic cardiac differences *in-situ,* displaying two different mechanisms of adaptation for male and female heart at increasing frequency. Male hearts tended to change their intrinsic spatiotemporal pattern of contraction/relaxation at the rising frequency of stimulation and to preserve their kinematics for all BCL. Female hearts denoted an opposite trend tending to preserve the spatiotemporal pattern and modify their kinematic response at different BCL. Our results are limited by the absence of the z-dimension and we recapitulate a 3D phenomenon in a 2D analysis. However, this precise and repeatable approach is useful to simplify the analysis without losing the main goal of the study and allow us to make comparison between several experimental sets. Future technological implementation will aim to introduce a second high-speed camera together with a PV-loop, a pressure catheter for peripheral resistance measurement and echocardiographic investigation for simultaneous acquisition of 3D epicardial kinematics and cardiac function.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbiomolbio.2019.05.003.

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