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Enhancing respiratory sinus arrhythmia increases cardiac output in rats with left ventricular dysfunction

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Abstract

Natural pacing of the heart results in heart rate variability, an indicator of good health and cardiac function. A contributor to heart rate variability is respiratory sinus arrhythmia or RSA – an intrinsic respiratory modulated pacing of heart rate. The loss of RSA is associated with poor cardiac prognosis and sudden cardiac death. We tested if reinstatement of respiratory modulated heart rate (RMH) would improve cardiac performance in heart failure. Heart failure was induced in Wistar rats by ligation of the left anterior descending coronary artery. Rats were unpaced, monotonic paced and RMH paced; the latter had the same average heart rate as the monotonic paced animals. Cardiac function was assessed non-invasively using echocardiography before and after two weeks of daily pacing at a time when pacing was turned off. RMH increased cardiac output by 20 ± 8 % compared to monotonic pacing (-3 ± 5 %; $P<0.05$). This improvement in cardiac output was associated with an increase in stroke volume compared to monotonic pacing ($P = 0.03$) and improvement in circumferential strain ($P = 0.02$). Improvements in ejection fraction ($P = 0.08$) and surrogate measures of left ventricle compliance did not reach significance. Increases in contractility ($P<0.05$) and coronary blood flow ($P<0.05$) were seen *in vitro* during variable pacing to mimic RMH. Thus, in rats with left ventricular dysfunction, chronic RMH pacing improved cardiac function through improvements in systolic function. As these improvements were made with pacing switched off, we propose the novel idea that RMH pacing causes reverse-remodelling.

Introduction

Respiratory sinus arrhythmia (RSA) is a cardiorespiratory phenomenon characterized by an increase in heart rate (HR) during inspiration and a fall during expiration (Anrep, Pascual et al. 1936, Eckberg 1983). From an evolutionary standpoint it is highly conserved being found throughout the animal kingdom from primitive cartilaginous fish (Taylor 1994). However, despite decades of studies on RSA its functional significance remains highly controversial (Larsen, Tzeng et al. 2010, Ben-Tal, Shamailov et al. 2014).

Some evidence suggests that RSA equalises cardiac output between the left versus the right side of the heart (Elstad 2012). Others have suggested that it optimizes pulmonary gas exchange through the close matching of ventilation and pulmonary artery blood flow (Hayano, Yasuma et al. 1996, Giardino, Glenny et al. 2003, Grossman and Taylor 2007). However, this theory is contentious (Sin, Webber et al. 2010) and a recent mathematical modelling study could not validate this finding (Ben-Tal, Shamailov et al. 2012). Instead, Ben-Tal and colleagues proposed that RSA reduced cardiac work while maintaining physiological levels of arterial carbon dioxide (Ben-Tal, Shamailov et al. 2014). We suggest that through these mechanisms RSA serves to optimize oxygen delivery to the heart with increasing workload (Elstad, O'Callaghan et al. 2018). In contrast, RSA is lost in cardiovascular and pulmonary diseases (e.g. heart failure, hypertension and chronic obstructive pulmonary disease) but whether this promotes the progression of heart disease is unknown. Certainly, a loss of RSA is a well described prognostic indicator for cardiovascular disease and sudden cardiac death (Binkley, Nunziata et al. 1991, Mortara, La Rovere et al. 1994, La Rovere, Bigger et al. 1998). Thus, in heart failure where there is already reduced coronary blood flow and oxygen supply, the absence

of RSA may further compromise adequate tissue perfusion enhancing susceptibility to episodes of hypoxia triggered by increased metabolic demand (Elstad, O'Callaghan et al. 2018) thereby promoting cardiac remodelling (Mulder, Barbier et al. 2004). The robust observation of a loss of RSA in cardiorespiratory diseases in addition to the predicted role of RSA in enhancing cardiac work efficiency has led to the specific question of whether there would be any therapeutic gain from its reinstatement in an animal model of heart failure.

Using a novel analogue biofeedback device, we demonstrated recently that RSA can be artificially augmented, at least acutely, by modulating cardiac pacemaker spike frequencies in phase with electromyographic activity of the diaphragm in healthy, anaesthetised rats (O'Callaghan, Chauhan et al. 2016). Here, we have deployed this device to drive RSA *chronically* in a conscious rat with induced heart failure. We tested the hypothesis that enhancing RSA in rats with heart failure will show an improvement in cardiac function by ventricular remodelling and that this will be superior to that seen with monotonic cardiac pacing. Our novel findings support the contention that RSA improves cardiac function in heart failure.

Methods

Ethical Approval

All animal experiments were performed in accordance with institutional guidelines and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the respective University of Bristol animal ethics committee.

Induction of heart failure

Heart failure was induced by myocardial infarction (MI) in male Wistar rats (280-300g) according to published methods (Pfeffer, Pfeffer et al. 1979, Francis, Weiss et al. 2001). Animals were anesthetized with ketamine (60 mg/kg, i.m.; Vetalar®, Boehringer Ingelheim,USA) and medetomidine (250 µg/kg, i.m.; Domitor®, Orion Pharma, UK), endotracheally intubated (after local swab application of 2% Xylocaine® HCl (AstraZeneca, UK)), and ventilated mechanically (70-85 breaths per minute, 1.5-2.0mL/breath; Advanced Safety Ventilator MA1 55-7059, Harvard Apparatus, USA). A left thoracotomy at the 4^{th} intercostal space was performed to expose the heart, and the left anterior descending coronary artery was ligated with polyester suture (4-0) between the pulmonary artery outflow tract and the left atrium. The thoracotomy was closed with suture and the rats recovered with atipamezole (2mg/kg s.c.; Antisedan®, Pfizer, USA), given Buprenorphine (30µg/kg i.m. Vetergesic®, CeVa, UK) for analgesia and Hartman's solution for rehydration as required. Approximately 7 days post- MI, rats were re-anaesthetised with isoflurane (5% induction, 1.8-2.0% maintenance via a nose cone) and the cardiac function was assessed using non-invasive echocardiography (see below) to select rats with a sufficient infarct.

Surgical implantation of EMG, ECG and pacing leads.

Rats with an ejection fraction ≤50% measured one week after MI were selected to undergo the second surgery for implantation of cardiac pacing and dEMG recording leads. A 6-channel pedestal with back-mount (Type 363 components, PlasticsOne, USA) contained percutaneous electrodes (2× cardiac ECG, 2× dEMG and 2× cardiac pacing) and remained externalised for tethering. Electrodes had socket-pin connections at the pedestal and stainless-steel suture-pads (~0.5mm^2) for electrical recording/stimulation (PlasticsOne, USA). For implantation, rats were anesthetized and mechanically ventilated as described above. Using aseptic techniques, the sterilised multi-lead electrode pedestal was sutured into place (nylon monofilament, 4-0, Ethilon, Ethicon®, USA) between the scapulae. The chest was opened on the right side between the 3rd and 4th intercostal space, the lungs retracted and the two cardiac pacing leads anchored at two positions using sutures (6-0 TiCron™ Braided Polyester Suture, Covidien, USA): (i) 2-3 mm from the electrode pads the leads were secured to the right ventricle via a small tear in the pericardium at an endocardial depth ≤0.5mm. (ii) pacing electrodes were sutured separately to the right atrium, maintaining an inter-electrode distance of 1-2mm. The electrode suture pads (contacts) were in direct contact with the myocardium. The 3rd intercostal space was closed with 3-4 sutures (4-0, Mersilk braided, Ethicon, USA). The leads exited the chest through the 4th intercostal space which was closed using a purse-string suture immediately after restoring negative intra-pleural pressure and spontaneous breathing restored. The leads were anchored extra-thoracically with suture to the 4th intercostal space. The two ECG leads were sutured (4-0, Mersilk braided, Ethicon, USA) via their suture pads subcutaneously at the 1st or 2nd intercostal space and the second at the xyphoid process. Finally, the recording leads were posted through an

incision in the abdominal muscle layer posterior to the last rib and sutured (4-0, Mersilk braided, Ethicon, USA) to the right-lateral costal diaphragm, as previously described (O'Callaghan, Chauhan et al. 2016). The abdominal muscular wall was closed and the overlying skin incision closed with sutures. All leads were tunnelled to the pedestal and connected to it. Prophylactic antibiotics (s.c, 2% Baytril®, Bayer Animal Health, UK), analgesics (s.c. Buprenorphine) and hydration solution (Hartman's solution i.p) were given to aid post-operative recovery. Atipamezole (s.c) was given to reverse anaesthesia and the rats were recovered under a heat lamp until mobile. Post-operative care was given according to veterinary guidelines and rats regained pre-operative body weight within 2-5 days. Rats were recovered for 2-3 weeks before experimental pacing was initiated.

Approximately 10 animals died between LAD ligation and the end of the study and all deaths occurred during or within 24 hours of this second surgery. A similar mortality rate from this surgery occurred in SHAM-MI rats in an earlier pilot study, indicative of a high-risk surgery.

Pacing protocol

Three to four days before the pacing protocol was initiated, rats were acclimatised to the tethering setup. During acclimatisation, dEMG and ECG were recorded (1401 acquisition hardware and Spike2 v8 software, Cambridge Electronic Design, UK) after amplification (both ×10k gain, 300-500Hz filtered; Model 1700 Differential AC Amplifier, A-M systems, USA) and the average resting heart was determined from their ECG. The pacing threshold was determined in each rat by recording an ECG whilst stimulating the right atrial electrodes at 450 bpm (Hz) and increasing the voltage until the rat heart beat was reliably paced (1.2 - 3.9 V). The heartbeat was

deemed paced when the QRS complex had a physiological latency from the stimulus artefact (or artificial P wave) of 55-65 ms.

At 4 weeks post-MI, pre-pacing cardiac function was assessed by echocardiography (described below). The following day daily pacing commenced (8 hours per day for two weeks) and set above their intrinsic HR at rest. Rats were divided into three groups: (i) respiratory-modulated heart rate paced (RMH; n=5) using our previously described biofeedback device (Nogaret, Zhao et al. 2013, O'Callaghan, Chauhan et al. 2016); (ii) monotonically paced (n=5) with an isolated, constant voltage stimulator (DS2A, Digitimer Ltd, UK) triggered by a TTL output generated by a digital-analogue converter output, controlled by Spike2 software; (iii) unpaced (n=7) for a time-matched control. The RMH waveform was tuned at the start of day 1 such that the minimum HR was above the average intrinsic HR at rest and the peak-trough amplitude of RMH was 20-30 bpm, with peak HR occurring with inspiration (Figure 1B, inset). The HR of the monotonic group was matched to the average HR of RMH-paced rats (Figure 1B, inset). Rats were paced as described for 14 days and no animals died during the pacing (or unpaced) protocol period.

The percentage of successful pacing was determined daily using a bespoke software code (Spike2, CED, UK) that enabled R-waves from the ECG recording to be counted from a post-stimulus triggered histogram triggered by the pacing voltage artefact (Figure 1C). The efficacy of pacing was calculated and based on the number of R-waves within 55-65 ms of the stimulus artefact relative to the total number of paced stimuli and multiplied by the duration of pacing to get a daily pacing efficacy. This was averaged over the 14 days of pacing and we set the average pacing efficacy cut off at 30 %, below which rats were excluded. This cut-off yielded the most similar amount of pacing between the monotonic and RMH paced groups.

Echocardiography

Analysis of cardiac function was conducted non-invasively using start of the art echocardiography and previously established methods (MKT02620 Imaging Guide, FUJIFILM VisualSonics Inc, The Netherlands)(Baily, Lehman et al. 1993, Lang, Bierig et al. 2005). Echocardiography was conducted at two time-points, on all animals under isoflurane anaesthesia (1.8%) while the pacing was off: (i) the day before pacing was initiated and (ii) the day after the 14th day of pacing. With pacing off we could fully test the hypothesis that RMH pacing improved cardiac function through therapeutic remodelling. Both motion (M)-mode and brightness (B)-mode views of the parasternal short axis (PSAX) and parasternal long axis (PSLAX) were taken in duplicate. B-mode, PSAX views were taken at 3 transverse locations, 2-3 mm posterior to the base, mid-papillary and 2-3 mm anterior to the apex of the heart. These 3 locations and the long-axis length (aortic valve to endocardial apex) were used for the Simpson's method of calculating end-diastolic and end-systolic LV volumes from which other cardiac function variables were calculated automatically by the software (Vevo LAB v3.1.0, FUJIFILM VisualSonics Inc, The Netherlands). These included cardiac output, ejection fraction, fractional area shortening and stroke volume. Heart rate was calculated from the RR interval on the concurrently recorded ECG waveform. The cardiac function variables of the PSAX views of the base, middle and apex were averaged for each rat and the values before and after 2 weeks of pacing (and 2 weeks unpaced) were compared and grouped. LV posterior wall diameters were calculated from the PSAX (B-mode), mid-papillary view (Figure 4A). Echocardiography analysis was not blinded but was repeated 3 months after the first analysis and raw data results were within the 3-9 % expected intra-user variability range (Stein, Tiwari et al. 2007) and the outcomes of grouped average

comparisons were consistent between both rounds of analysis. Data from the second round of analysis was used for statistical analysis and reporting.

Cardiac strain analysis (Speckle-tracking echocardiography)

Changes in cardiac strain have previously been reported as occurring early in the cardiac remodelling process in mouse models of myocardial infarction (Yamada, Arrell et al. 2013, Bhan, Sirker et al. 2014). Therefore, VisualSonics VevoStrain software was used on images previously acquired using echocardiography to assess the effect of the pacing paradigms on cardiac contractility using established methods (Bhan, Sirker et al. 2014, Pedrizzetti, Mangual et al. 2014, Kovács, Oláh et al. 2015). B-mode images of the PSAX views over 3 consecutive heart beats during expiration, at 3 transverse locations (base, middle, apex) were analysed using the VevoStrain software, before and after two weeks of pacing. Endocardial walls were traced and the software algorithms automatically calculated radial and circumferential peak strain (expressed as % thickness relative to its original length), where increased contractility means more positive radial strain and more negative circumferential strain (Dandel, Lehmkuhl et al. 2009, Bhan, Sirker et al. 2014). Each transverse section was divided into 6 equal segments (Figure 3A) and the data grouped to include the anterior free wall, lateral wall, posterior wall and inferior free wall. The posterior septal wall and anterior septum were excluded from analysis because they were obscured by the sternum to a variable degree in different rats. The anterior free and lateral walls were predominantly infarcted whilst the posterior and inferior free walls were non-infarcted.

B-mode images of the PSLAX were also analysed using VeVoStrain software to calculate peak radial and longitudinal strain, strain rate and time to peak strain

(TTP). The standard deviation of TTP strain was calculated as a measure of cardiac dyssynchrony (Yamada, Arrell et al. 2013).

Histological analyses

After the final echocardiography measurements rats were deeply anaesthetised with sodium pentobarbital (200 mg/kg i.p.) and perfused retrogradely via the ascending aorta with isotonic saline supplemented with 0.2M KCl to arrest the heart in diastole, followed by fixation with 4% paraformaldehyde in phosphate buffered saline. The heart was excised, stored in fixative for 2 hours (4 °C) and then 30% sucrose in phosphate buffered saline overnight. The tissue was cut coronally into 4 equal segments and each segment was paraffin embedded and cut at 7μm thickness onto gelatin-coated slides. Alternate sections were stained with Masson's Trichrome and Picrosirius Red and infarct size calculated from an average of 12 sections per animal using planimetry and public-domain software (NIH ImageJ, USA). The perimeter length of the infarcted myocardium was expressed as a percentage of the total left ventricle circumference. Collagen infiltration was quantified from picrosirius red stained sections (20 images per rat) using polarised light according to standard methods. Two polarising filters were used to cause birefringence of collagen fibres to distinguish type I and III collagen and photographed (Leica DM750 microscope with Leica Hi Plan 40x objective and a ICC50W camera). Collagen fibres, appearing green and orange under polarised light were quantified using public-domain software (NIH ImageJ, USA) and expressed as the percentage of total myocardial area per section.

Spectral analysis

ECG recordings were made in each treatment group while rats were tethered and resting on day 0 before pacing and day 14 after pacing, while pacing was switched

off. Segments of ECG data were imported into LabChart (version 7, ADInstruments, AUS) at 1kHz and 5min periods of stable heart rates were analysed with the in-built HRV software according to standard settings for rat: 150 ms < normal RR interval range < 218 (ectopic beats excluded), 1 ms histogram bin size, variation threshold (deltaNN) >16 ms, 512 FFT, Hann window, 1/2 overlap and frequency bands: VLF 0-0.27 Hz, LF 0.27-0.75 Hz, HF 0.75-3.3Hz (Waki, Katahira et al. 2006).

Langendorff preparation to investigate coronary flow and LV contractility

The effects of RMH versus monotonic heart rates on coronary flow and LV contractility were evaluated in Langendorff heart preparations. Male Wistar rats (280-300g; n = 7) were anesthetized with isoflurane (5%) and the heart was quickly removed and immersed into ice cold Krebs-Henseleit buffer containing (mM): 118 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 11 glucose and 95% O_2 and 5% CO_2 at 37 °C (pH 7.4). After aortic cannulation, the heart was perfused with constant pressure (70-80 mmHg) and coronary flow measured by collecting the effluent. Coronary perfusion pressure was measured through a side branch of the perfusion cannula connected to a pressure transducer (ADInstruments, AUS). The left ventricular pressure was measured with a water-filled balloon inserted into the left ventricle and connected to a second pressure transducer. The balloon volume was modified through a stopcock attached to the ventricular pressure transducer using a syringe to maintain a left ventricular diastolic pressure of 5–10 mmHg. Coronary perfusion and ventricular pressure signals were recorded using Power Lab/4SP acquisition hardware and LabChart software (both ADInstruments, AUS). HR and left ventricular contractility (+dP/dt - maximal slope of the systolic pressure increment) were calculated from the ventricular pressure pulses. Bipolar electrodes were placed into the right atrium for electrical stimulation of the sinoatrial node.

Pacing was produced by an isolated constant voltage stimulator (DS2A, Digitimer Ltd, UK) coupled to a computer and the software Spike 2 (Cambridge Electronic Design, UK) which was used to control the stimulation. Two patterns of stimulation were performed on each heart; monotonic and simulated RMH. The simulated RMH had a duty cycle of 0.4s high-HR and 0.6s low-HR, where the low HR was 10bpm above baseline and the high HR was 30-50bpm above the low HR. Monotonic pacing was the average of the low and high HR. Stimulus pulses were 1 ms wide and 3-5V amplitude. The stimulations were performed under β-adrenergic receptor blockade, atenolol (15mM), to avoid sympathetic stimulation. The blockade effectiveness was tested by isoproterenol (20nM). Pacing was performed 20-40 min after left anterior descending coronary artery occlusion. At the end of the experiment, the hearts were perfused with Evans' blue to allow quantification of the infarct size.

Statistical analysis

Paced HR and pacing efficiency were compared with a student's t test and 95% confidence interval where $\alpha = 0.05$. Changes in cardiac function were assessed by first normalising the value in each rat after two-weeks of treatment to the value before treatment. Normalised values were averaged within treatment groups and a one-sample t test (hypothetical mean = 0, $\alpha = 0.05$) was used to test for a difference to null effect. Between group differences were first assessed using a one-way ANCOVA, where change was the dependent variable and baseline the covariate. If the covariate was not the source of variation, a one-way ANOVA followed by post hoc Bonferroni's t test for multiple comparisons was used to compare data between groups.

The effect size and 95% confidence intervals of each treatment on the measures of cardiac function was calculated using Cohen's d method (Cohen 1988) to assess the

magnitude of mean difference in cardiac function with treatment. An effect size of 0.2, 0.4 and 0.8 are considered small, medium and large changes in magnitude respectively (Cohen 1988).

Results

Exclusion criteria and group numbers

37 rats with MI were studied. Prior to the first day of pacing, sensing from the right-atrial pacing leads was used to record the electrical activity at the right atrial pacing lead site to infer the presence/absence of atrial contact. Rats in which atrial contact was poor and insufficient for atrial pacing formed the unpaced group (n=7). Rats in the pacing groups that were successfully paced for < 30% of the daily pacing duration (averaged over 14 days) were excluded (n=6). Rats with an infarct size <30%, determined histologically, were also excluded from further analysis (n=12). Thus, 19 rats completed the study comprising 7 unpaced, 6 RMH- and 6 monotonically- paced.

Efficacy of pacing and its variation in the RMH group

The resting HR of conscious rats before pacing was 343 ± 7 bpm and 333 ± 8 bpm for the monotonic and RMH groups respectively. Therefore, rats were paced above this rate to ensure most of the pacing duration was above intrinsic HR. The daily duration of effective pacing was not significantly different for monotonic- (44% effective, 2.7 ± 0.2 hours) versus RMH (50% effective, 3.5 ± 0.6 hours) paced rats (P=0.31). The mean paced HR was not different between monotonic- and RMH-paced groups (396 ± 6 bpm and 399 ± 7 bpm respectively, P=0.90). As expected, the within-rat standard deviation of paced HR (i.e. its variance) was different between monotonic- and RMH- paced groups (0 ± 0 bpm and 15 ± 2 bpm respectively, P = 0.0001).

Improvement in cardiac function after RMH pacing

Both prior to pacing being initiated and at the end of the 2-week pacing period, data were obtained by echocardiography in anaesthetised rats with pacing switched off. These data for each treatment group are shown in Table 1. With pacing off, intrinsic HR during echocardiography at the end of 2 weeks pacing were: 407 ± 9 bpm for unpaced, 381 ± 11 bpm for monotonic and 411 ± 9 bpm for RMH; these were not different. In the RMH group cardiac output was greater than that in the monotonic paced rats (23 ± 6 % vs. -3 ± 5 %;Figure 2A, $P<0.05$). The cardiac index, which corrects for differences in body weight, was numerically (but not statistically) higher in RMH versus monotonic paced rats (Figure 2A, $P = 0.05$). RMH paced rats had a significant increase in cardiac output ($P=0.03$) but not cardiac index ($P=0.17$) when compared to pre-pacing treatment values (Paired T-test). In monotonic paced rats, the cardiac and stroke volume indices were decreased compared to their baseline values (-12 ± 4 % and -13 ± 5 % respectively, $P< 0.05$).

Further interrogation of cardiac function indicated that the relative increase in cardiac output observed after 2 weeks RMH vs monotonic pacing was more likely due to an numeric increase in stroke volume (25 ± 8 % vs. -5 ± 5 %; Figure 2B) and not heart rate, as HR was not different between treatment arms (Figure 2C). However, statistical analysis of stroke volume (using ANCOVA) suggested that the baseline value contributed to the source of variation ($P = 0.03$) and therefore between-group comparisons would not be appropriate for this variable. All measured cardiac variables were tested using the ANCOVA model to identify any interactions with baseline values that may have contributed to differences between RMH- and monotonic- paced rats. The results are shown in table 2. The relative increase in stroke volume of RMH vs monotonic paced rats likely reflects a difference in ejection

fraction (11 ± 6 % vs. -9 ± 7 %, P = 0.09; Figure 2D) since left ventricular end-diastolic volume (LVEDV) did not change in RMH vs monotonic-paced rats after 2 weeks of pacing (Figure 2E). Ejection fraction and LVEDV were not different in RMH- or monotonic- paced rats when compared to unpaced rats. The cardiac function data collected before and after 2 weeks, averaged within treatment groups, is shown in Table 1.

Whilst RMH did not induce a statistically significant difference in ejection fraction and LVEDV (within groups), an examination of the effect size of RMH and monotonic pacing on the mean difference in these variables (shown underneath the respective columns in Figure 2) suggests that RMH pacing had a moderate positive effect on ejection fraction (effect size 0.35) where monotonic pacing had a moderate negative effect (effects size -0.32). RMH pacing also had a moderate-strong, positive effect on LVEDV (effect size 0.77). The mean difference over time of each cardiac variable, averaged within treatment groups and calculated effect sizes with confidence intervals are given in supplementary table 1.

Chronotropic analysis

There was no difference in resting basal HR (whilst pacing was off) between the 3 groups before or after 2 weeks of pacing treatment as recorded under anaesthesia (Two-way ANOVA with replication, $P > 0.05$, $n = 10$, data not shown). Thus, HR did not change after 2 weeks of pacing (or unpaced) in any group. Spectral analysis of the R-R interval showed no change within or between groups in the LF, HF oscillations or LF: HF ratio after 2 weeks of pacing (data not shown). This suggests pacing had no effect on parasympathetic cardiac tone or cardiac autonomic balance over 2 weeks of pacing (or unpaced).

Potential mechanisms for the improvement in cardiac function with pacing

(i) Systolic function

Systolic function was assessed using myocardial wall strain analysis. Circumferential strain became more negative after 2 weeks of RMH pacing, indicating improved circumferential contractility in this group (-33 ± 9%, P = 0.01 compared to null effect; Figure 3C). There was no change in circumferential or radial strain within the unpaced or monotonic- paced treatment groups (compared to null effect). There was no difference in radial or circumferential short axis strain *between* treatment groups (Figure 3C). There were no differences within or between groups in the improvement (or decline) in cardiac dyssynchrony (time to peak strain (T2P)) or in global longitudinal strain or strain rates (Figure 4D-F).

(ii) Diastolic function

LVED posterior wall (LVEDPW) thickness (indicated in Figure 5A) is strongly correlated with myocardial wall stiffness and can be used in the absence of LVED pressure (LVEDP) data to estimate diastolic function (Grossman, McLaurin et al. 1974). LVEDPW thickness during diastole, measured using echocardiography, tended to increase after 2 weeks in the unpaced group (1.82 ± 0.09 mm vs 1.95 ± 0.03 mm, P=0.11), but was unchanged after 2 weeks of RMH- (1.95 ± 0.12 mm vs 1.82 ± 0.07 mm, P=0.12) or monotonic- (1.81 ± 0.09mm vs 1.84 ± 0.13mm, P=0.34) pacing. There was no difference in effect of treatment groups on LVEDPW thickness (Figure 5B). The proportion of collagen in the myocardium, determined histologically, was not different between the three groups after 2 weeks of pacing (unpaced, 5.3 ± 0.8%, n = 4; monotonic, 5.3 ± 0.9%, n = 4; RMH, 5.1 ± 0.8%, n = 5; Figure 5E), suggesting no detectable reduction in fibrosis from either pacing paradigms over this time frame.

Coronary flow and LV contractility analysis

The effects of RMH on coronary flow and LV contractility was evaluated in isolated hearts before and after MI. *In vitro*, the left anterior descending coronary artery occlusion produced an extensive MI (55 ± 3%, Figure 6B). Acute simulated RMH pacing increased coronary flow (10 ± 3%), LV contractility (dP/dt_{max} 8 ± 3%) and LV relaxation (dP/dt_{min} 11 ± 4%)). Monotonic pacing increased coronary flow (14 ± 3%), dP/dt_{max} (9 ± 2%) and dP/dt_{min} (11 ± 4%) to the same magnitude as simulated RMH (Figure 6A, C - E).

Discussion

The major finding of the present study indicates that, for the primary comparison, RMH increased cardiac output compared to monotonic-pacing in rats with heart failure after two weeks of pacing. Further, the improvement in cardiac output observed after 2 weeks of RMH pacing was with pacing switched off. Given this, we suggest that the improvement in cardiac output reflects a process of reverse-remodelling in rats with left ventricular dysfunction.

There is an abundance of evidence demonstrating RSA is diminished or absent in a range of chronic diseases, including cardiovascular disease and heart failure. However, the function of this physiological phenomenon is yet to be fully established. The proposed benefits of RSA include stabilisation of blood pressure, optimisation of cardiopulmonary gas exchange and increased cardiac energy efficiency (Hayano, Yasuma et al. 1996, Giardino, Glenny et al. 2003, Elstad 2012, Ben-Tal, Shamailov et al. 2014). Any one of these would assist patients with heart failure where myocardial function and energy are suboptimal. In addition, existing pacemakers do not apply within-breath modulation of heart rate and therefore, are more like the monotonic treated rat group in this study. Until now it was not possible to chronically reinstate RSA and investigate its potential therapeutic effects in a conscious animal with heart failure. This study is the first to have used a novel pacemaker with physiological biofeedback to investigate if reinstating RMH improves cardiac function in rats with left ventricular dysfunction beyond that achieved with conventional, monotonic pacing.

Caveats

Both monotonic and RMH pacing captured the heart rate for an equivalent amount of time each day that was less than the full duration of the tethered pacing period (44 and 53% for monotonic and RMH respectively). This was in most part due to intermittent grooming, eating and sniffing behaviour of the rat that drove heart rate above the pacemaker set point. This resulted in rats being actively paced for only ~3-4 hours each day, a relatively short duration compared with patients paced in clinical practice. However, it is thought that the process of cardiac remodelling is similar in process but occurs more rapidly in rats than in humans (Goldman and Raya 1995, Cleutjens, Blankesteijn et al. 1999). In addition, a novel study demonstrated that 6 hours a day of pacemaker-induced asynchrony in canines with heart failure resulted in significant improvements in cardiac function (Kirk, Chakir et al. 2015).

As demonstrated in our previous study, the RMH waveform was consistent with the RSA waveform reported in conscious, healthy rats (Bouairi, Neff et al. 2004), whereby HR increased during inspiration and decreased during expiration (O'Callaghan, Chauhan et al. 2016). We did not investigate the long-term effect of pacing with different waveform patterns, such as inverse RSA or non-respiratory frequencies of heart modulation; and this awaits future investigations.

The relative increase in cardiac output observed in this study was driven by an increase in stroke volume without a change in heart rate. Stroke volume can be increased by an elevation in ejection fraction where LVEDV is maintained (increased contractility) or by enhanced filling where ejection fraction is maintained. The latter is caused by increased diastolic capacity arising from better myocardial compliance. In

this study, whilst the increase in ejection fraction did not reach significance in RMH-paced rats, there was a moderate, positive effect size of RMH on ejection fraction. In monotonic-paced rats, ejection fraction did not reach a significant change from baseline, but there was a moderate effect size of monotonic pacing with *lower* ejection fraction. Taken together, this suggests there was a tendency for RMH pacing to increase the ejection fraction compared to monotonic pacing. In addition, circumferential peak strain was significantly improved by RMH pacing suggesting an improvement in contractility of the recovering myocardium. Since circumferential peak strain is negatively associated with MI severity (Bhan, Sirker et al. 2014), the latter finding supports the notion that RMH pacing reverses the pathological remodelling in heart failure. However, these results were not found consistently with other measures of cardiac strain analysis and a larger cohort of rats is likely required to address this.

RMH pacing may have also prevented deterioration in diastolic function in rats with left ventricular MI. It is known that myocardial compliance decreases over time in the rat model used herein because of increased interstitial collagen infiltration (Whittaker, Kloner et al. 1994, Milanez, Gomes et al. 1997). Whilst it was not technically possible to measure LVEDP in our rats in the conscious state, we did measure LVEDPW thickness and observed a decrease in the unpaced rats, suggesting worsening compliance over the 2-week period (i.e. weeks 4 to 6 post-MI). However, in the RMH paced rats, LVEDPW was thicker suggesting the decline in compliance was stalled. In addition, whilst LVEDV was not significantly increased in RMH or decreased in monotonic groups, there was a moderate to large effect size of RMH increasing LVEDV. However, there was no difference in interstitial collagen infiltration, suggesting that any improvement in LV myocardial compliance is unlikely

to have driven the increased cardiac output in RMH-paced rats. On balance, the relative increase in cardiac output could be due to the synergistic interaction between subtle improvements in both diastolic and systolic function. However, the evidence to date points in favour of improving systolic function driving the therapeutic benefit of RMH compared to monotonic pacing. Further studies are required to determine the longevity of the RMH-induced increase in cardiac function after pacing is withdrawn.

Novel insights into the function of RSA

Recent mathematical modelling of RSA suggested it could conserve cardiac energy whilst maintaining $PaCO_2$ (Ben-Tal, Shamailov et al. 2012). Improved cardiac efficiency is contingent on decreased myocardial oxygen demand and this could be achieved by either optimisation of coronary blood flow and/or intracellular calcium bioavailability. Coronary blood flow is decreased in experimental heart failure and cannot match demand (Spinale, Tanaka et al. 1992). Because it is not technically possible to measure coronary artery flow in real time in conscious rats, we paced isolated perfused in vitro working hearts. RMH increased coronary flow before and after MI to the same extent as monotonic pacing, and this correlated to heart rate reflecting an intrinsic matching of blood flow to work load. Similarly, LV contractility was increased during both RMH and monotonic pacing after MI. There is known to be a dynamic relationship between intracellular calcium transients and the preceding diastolic duration in isolated cardiomyocytes (Frampton, Orchard et al. 1991); however, the summative effect on cardiomyocyte contractility from pacing multiple, successive contractions with inter-beat variability versus a monotonic protocol is less clear. One study in rats demonstrated that pacing isolated cardiac trabeculae at an

average of 8Hz with a 40% variation in inter-beat interval generated 10% greater contractile strength than when pacing at the same average frequency without inter-beat variability (Torres, Varian et al. 2008). Interestingly, this effect was not observed at lower frequencies (4Hz or 6 Hz) in the same study and in another study, using the same protocol on cardiac trabeculae isolated from rabbit and canine; no difference was observed between monotonic and variable inter-beat interval pacing at the same average frequency in either species (Torres and Janssen 2011). It is worth noting that the variability employed in these studies was random, and thus more relevant to a situation of pathological arrhythmia than a physiological rhythm such as RMH.

Despite the beneficial effects achieved with RMH *in vitro*, we acknowledge that the isolated perfused heart lacks: (i) central autonomic innervation which, in part, has respiratory modulated activity (McAllen and Spyer 1978, Boscan, Allen et al. 2001) and contains vasodilatory vagal fibres innervating the coronary circulation (Feigl 1983), which may be important in increasing coronary blood flow on a breath by breath basis; and (ii) respiratory phasic changes in both intra-thoracic pressure and venous return which may both function to enhance coronary blood flow and increase pre-load for optimal cardiac filling; the latter would engage the Starling mechanism to increase contractility. These physiological mechanisms may all contribute to the beneficial effects of RMH versus monotonic pacing. Given this, we propose that future studies be conducted in conscious large animal models *in vivo* where coronary flow and left ventricular pressure can be measured in real time chronically with the different pacing paradigms. This would also allow an assessment of whether additional benefit can be obtained by pacing for longer than two weeks.

Another technique to investigate whether RMH pacing improved myocardial function may have been to measure oxygen levels within a coronary artery. This is not feasible in small animals, however arterial oxygenation from a femoral artery can be used. Whilst we did not measure arterial blood gases in the rats in this study, we did measure arterial blood gases in an earlier pilot study. In the pilot study, peripheral blood gases were measured before and after 45 min of RMH and monotonic pacing in anaesthetised rats 2 weeks post-LAD ligation and we found no difference in PaO2 or PaCO2 with either treatment (E. O'Callaghan, R. Lataro, JFR Paton – unpublished findings). However, this could have been confounded by anaesthesia and/or relatively short duration of pacing. Future studies in a larger animal model are required to adequately address this question.

Clinical relevance

In the UK alone, over 31,000 pacemakers are implanted per year to alleviate the symptoms of sinus node disease or AV block (Brignole, Auricchio et al. 2013, Edwards, Karner et al. 2015). All pacemakers function monotonically and some increase heart rate over successive breaths in response to the detection of increased movement or minute ventilation (Coman, Freedman et al. 2008). Existing pacemakers are unable to apply within-breath modulation of heart rate such as RMH. To date, no clinical trial has investigated whether cardiac function is compromised by the monotonic nature of existing cardiac pacemakers. There is, however, substantial evidence demonstrating that persistent ventricular asynchrony within cardiac contractions, caused by single-chamber cardiac pacing, is associated with a decline in ejection fraction and development of cardiomyopathy (Khurshid, Epstein et al. 2014, Bellmann, Muntean et al. 2016) and increased hospitalisations

for heart failure (Sweeney and Hellkamp 2006). Cardiac resynchronisation therapy, whereby left and right ventricles are paced in synchrony, aims to better emulate a physiological cardiac contraction and has demonstrated success in reversing the single-chamber pacing-induced cardiomyopathy (Eldadah, Rosen et al. 2006, Leclercq, Cazeau et al. 2007, Bellmann, Muntean et al. 2016, Khurshid, Obeng-Gyimah et al. 2018). Interestingly, it has been observed in a clinical setting, that the functional improvements driven by CRT are more pronounced in individuals starting with asynchronous heart failure (Kirk and Kass 2013), suggesting the restoration of synchrony has stronger biological effects than continuation of synchrony in heart failure. This phenomenon has since been demonstrated a canine model of heart failure (Kirk, Chakir et al. 2015) and raises the question in this study; is it the bouts of variability that are driving the observed changes in cardiac function? In this study, we observed that 2 weeks of monotonic pacing decreased cardiac and stroke volume indices, suggesting monotonic pacing was detrimental to cardiac function. The underlying mechanism for this effect needs further investigation as we were unable to demonstrate significant changes in the measures of diastolic or systolic function that were possible in this study.

Conclusion

We observed an increase in cardiac output in rats with left ventricular diastolic function after 2 weeks of pacing with a RMH but not with a conventional monotonic paradigm. The precise mechanism behind our novel observation requires further elucidation in fully instrumented large animals. However, the results presented herein are a tantalising demonstration of the physiological relevance of RSA.

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Disclosures

The authors have no conflicts of interest to declare.

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Table 1: Cardiac function determined by non-invasive echocardiography before and after 2 weeks of treatment with the specified cardiac pacing.

	Unpaced		Monotonic		RMH	
	Before	After	Before	After	Before	After
Weight (g)	301 ± 8	329 ± 10	296 ± 13	326 ± 12	303 ± 6	334 ± 8
CO ($mL.min^{-1}$)	116 ± 8	123 ± 5	118 ± 11	114 ± 11	114 ± 12	135 ± 12*
HR (bpm)	406 ± 8	407 ± 9	375 ± 11	381 ± 10	415 ± 6	411 ± 9
EF (%)	38 ± 4	40 ± 3	36 ± 2	33 ± 4	44 ± 4	47 ± 4
SV (mL)	287 ± 20	304 ± 14	312 ± 21	297 ± 26	276 ± 24	335 ± 20*
FS (%)	9 ± 3	12 ± 4	10 ± 1	10 ± 2	12 ± 2	14 ± 3
FAC (%)	32 ± 12	33 ± 12	28 ± 2	26 ± 2	36 ± 3	37 ± 4
LVEDV (mL)	768 ± 44	793 ± 67	863 ± 58	925 ± 104	649 ± 34	727 ± 42
LVESV (mL)	481 ± 53	490 ± 63	551 ± 46	628 ± 105	373 ± 39	392 ± 54
Cardiac index ($mL.min^{-1}.kg^{-1}$)	387 ± 24	375 ± 10	396 ± 31	349 ± 33*	375 ± 25	415 ± 30
SV index ($mL.kg^{-1}$)	954 ± 63	922 ± 20	1053 ± 66	916 ± 84*	906 ± 67	1009 ± 69

Abbreviations: CO, cardiac output; EF, ejection fraction; FAC, fractional area contraction; FS, fractional shortening; HR, heart rate; LVEDV, left ventricle end-diastolic volume; LVESV, left ventricle end-systolic volume; SV, stroke volume.

* $P<0.05$ compared to before treatment, paired student's t test.

Table 2: Results of ANCOVA test of measured cardiac variables testing for the interaction of baseline values with change from baseline for RMH- versus monotonic paced rats. Results of the Bonferroni t-test for multiple pairwise comparisons are also shown where appropriate. Abbreviations: see Table 1 and, CI, cardiac index; EDV, end diastolic volume; ESV, end systolic volume cardiac output; SVI, stroke volume index.

	ANCOVA				
Variable	Interaction (baseline x Group change)	Treatment difference of adjusted means	Covariate as source of variation? P-value (Y/N)	ANOVA (group sig)	Bonferroni t-test[1]
CO	P = 0.270	P = 0.045	P = 0.066 (N)	P = 0.043	P = 0.040
SV	P = 0.369	P = 0.122	P = 0.025 (Y)	n/a	n/a
EF	P = 0.244	P = 0.079	P = 0.502 (N)	P = 0.079	P = 0.093
FAC	P = 0.685	P = 0.094	P = 0.184 (N)	P = 0.069	P = 0.215 (P = 0.090^)
FS	P = 0.286	P = 0.658	P = 0.626 (N)	-	-
EDV	P = 0.347	P = 0.553	P = 0.347 (N)	-	-
ESV	P = 0.058	P = 0.697	P = 0.168 (N)	-	-
HR	P = 0.688	P = 0.927	P = 0.163 (N)	-	-
CI	P = 0.084	P = 0.079	P = 0.052 (N)	P = 0.052	P = 0.050
SVI	P = 0.069	P = 0.213	P = 0.022 (Y)	n/a	n/a

Figures

Figure 1 Experimental setup and representative example of HR waveforms during monotonic- and RMH- pacing

Heart failure rats with surgically implanted right atrial pacing and dEMG recording electrodes were tethered within their cages and connected to the custom-built device (A). The dEMG signal, consisting of bursts of activity during inspiration, was the bioelectric input to the device. The signal was amplified, integrated and processed by the device to generate a spiking electrical output that increased in frequency during inspiration. This device output was connected to the right atrial pacing leads to control the rat's heart rate. (B) Representative example of transverse cardiac sections from a heart failure rat stained with Masson's Trichome for infarct quantification. (C) A representative example from a rat being continuously paced by the device. The bottom channel shows dEMG bursts of activity that drive the device spike output and produce fluctuations in the instantaneous frequency of the device output (device-HR). The rat's ECG is recorded simultaneously and the rat's HR, calculated from the R-R interval is shown in red and perfectly matches the device-HR during effective pacing. An expanded segment (inset panel) shows that peak-HR occurs during inspiration, consistent with the published pattern of intrinsic RSA (Bouairi, Neff et al. 2004). A green line in the device-HR channel represents average-HR matched monotonic pacing. (D) The ECG waveform channel was used to calculate the latency of the rat's R-wave after the device (or monotonic) stimulus artefact was calculated for every stimulus spike and used to generate a post-stimulus triggered histogram of each day's pacing (bottom panel).

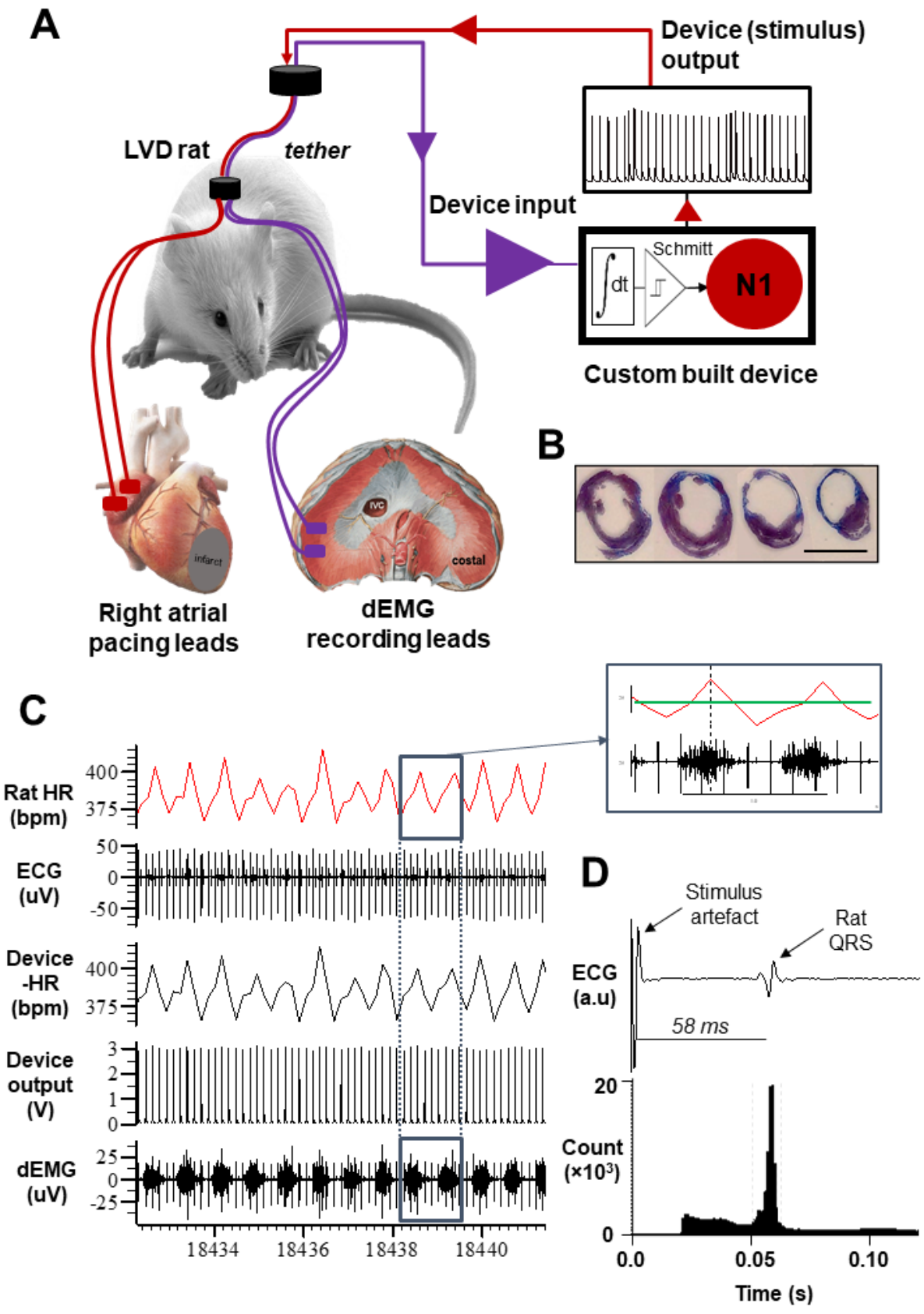
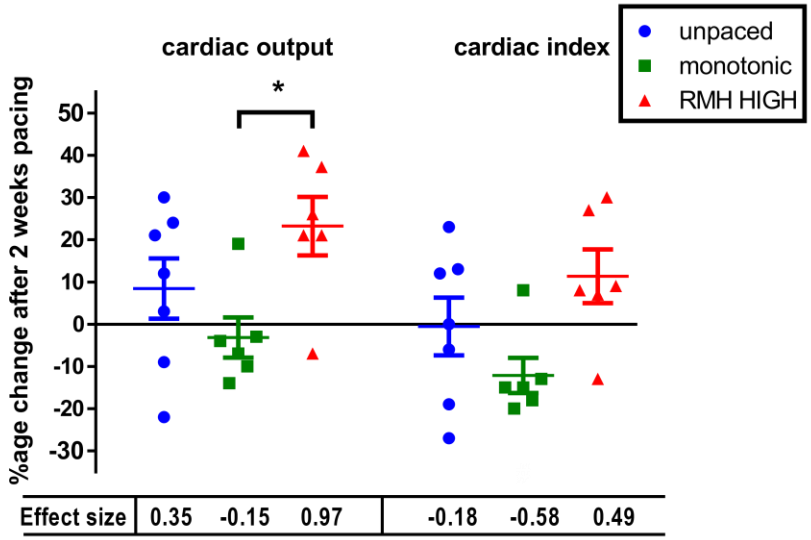
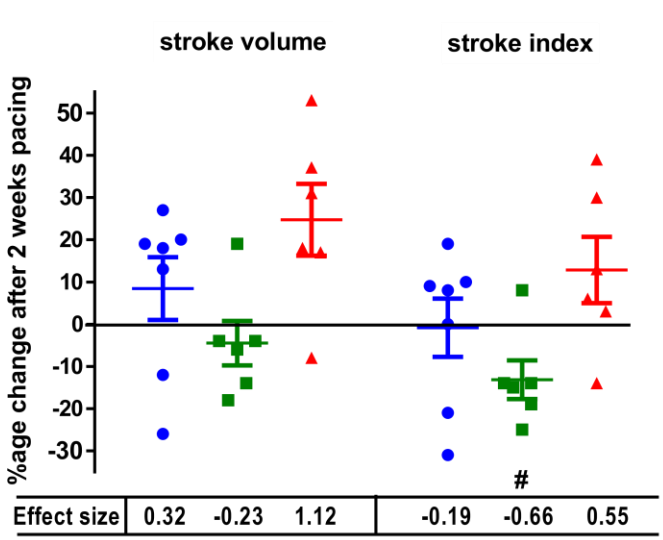

A
Device (stimulus) output
LVD rat
tether
Device input
Schmitt
dt
N1
Custom built device
B
IVC
infarct
costal
Right atrial pacing leads
dEMG recording leads
C
Rat HR (bpm)
400
375
ECG (uV)
50
0
-50
Device -HR (bpm)
400
375
Device output (V)
3
2
1
0
dEMG (uV)
25
0
-25
18434
18436
18438
18440
D
Stimulus artefact
Rat QRS
ECG (a.u)
58 ms
20
Count (×10³)
0
0.0
0.05
0.10
Time (s)

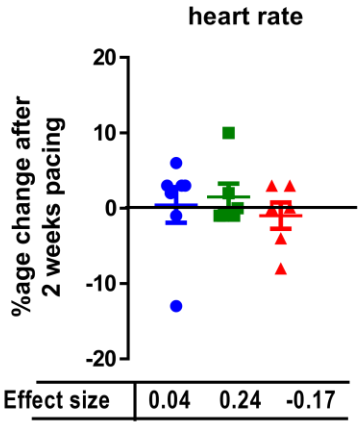
Figure 2 Changes in cardiac function after 2 weeks of monotonic or RMH pacing

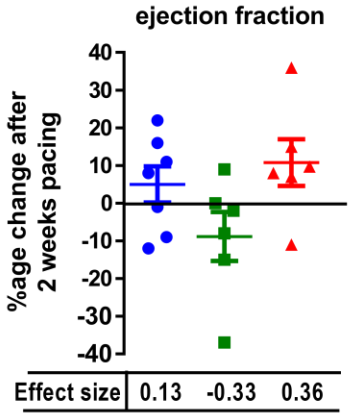
Cardiac output (and cardiac index) (A) and stroke volume (and stroke index) (B) increased significantly more in heart failure rats that underwent 2 weeks of RMH pacing compared to monotonic pacing, but not time-matched unpaced rats. Two weeks of RMH-pacing tended to increase cardiac output and monotonic pacing significantly decreased cardiac- and stroke-indices when compared to null effect. Heart rate (C), ejection fraction (D), end-diastolic and end-systolic left ventricular volume (E) did not change significantly over the 2-week pacing (and unpaced) period in any groups. The effect size for each treatment and variable are given below the x-axis.

Data are mean ± SEM. ***** $P < 0.05$ RMH compared to monotonic group (Unpaired t-test), # $P < 0.05$ compared to null effect (One-sample t test). P value is for comparisons to null effect.


cardiac output
cardiac index
unpaced
monotonic
RMH HIGH
%age change after 2 weeks pacing
*
50
40
30
20
10
0
-10
-20
-30
Effect size
0.35
-0.15
0.97
-0.18
-0.58
0.49


stroke volume
stroke index
%age change after 2 weeks pacing
#
50
40
30
20
10
0
-10
-20
-30
Effect size
0.32
-0.23
1.12
-0.19
-0.66
0.55


heart rate
%age change after 2 weeks pacing
20
10
0
-10
-20
Effect size
0.04
0.24
-0.17


ejection fraction
%age change after 2 weeks pacing
40
30
20
10
0
-10
-20
-30
-40
Effect size
0.13
-0.33
0.36

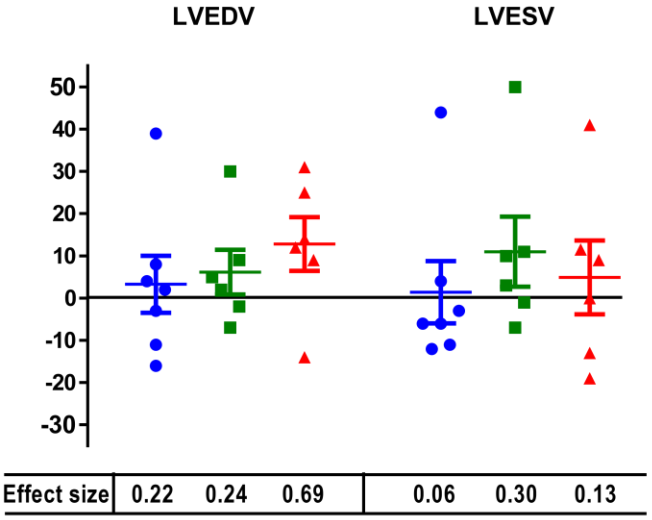

LVEDV
LVESV
50
40
30
20
10
0
-10
-20
-30
Effect size
0.22
0.24
0.69
0.06
0.30
0.13

Figure 3 Change in regional, endocardial strain (systolic function) after 2 weeks of monotonic or RMH pacing

(A) A representative example of a parasternal LV short axis, middle view with the endo- and epi-cardial walls outlined and divided into the 6 myocardial wall segments used in strain analysis, labelled in the adjacent schematic. (B) Representative example of radial (top) and circumferential (bottom) strain over a cardiac cycle. The coloured traces indicate the magnitude of radial or circumferential strain as a continuum of time and 6 locations along the endocardial wall (C) The relative changes in myocardial radial and circumferential peak strain, averaged over regions 1-4 of the endocardial wall are shown for each pacing treatment group.

Data are mean ± SEM. * is $P < 0.05$ for comparisons before treatment data.

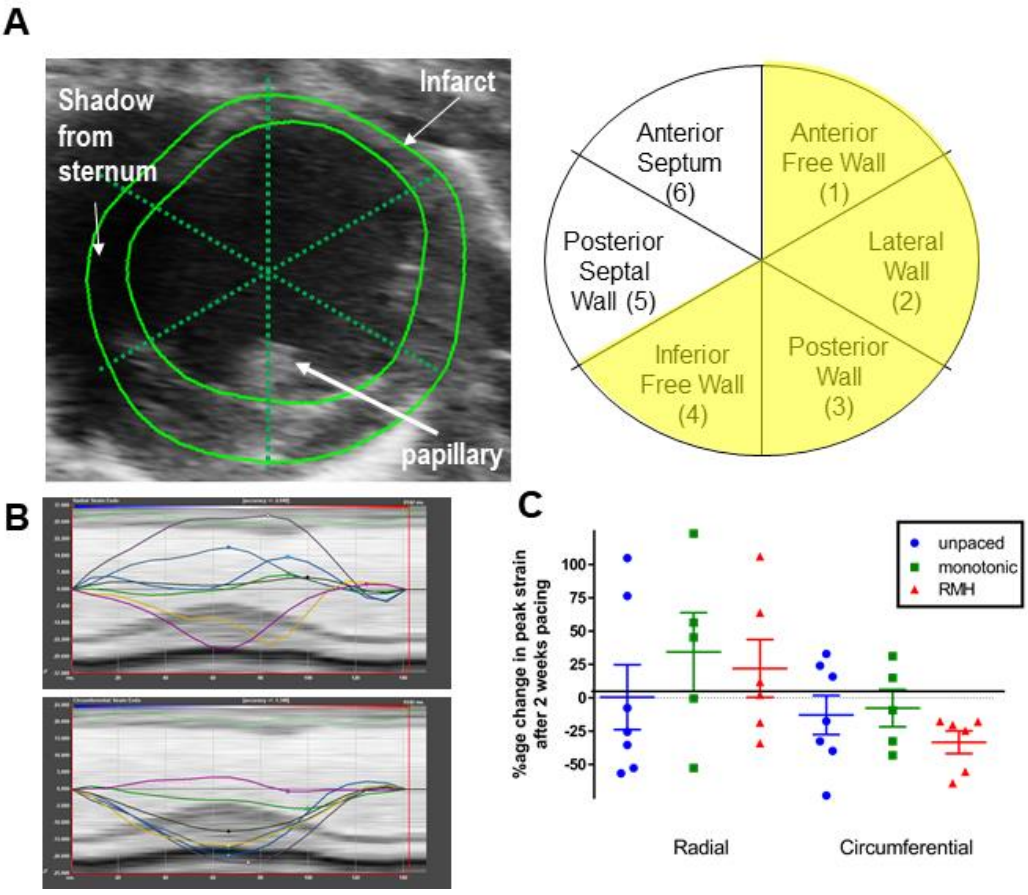

A
Shadow from sternum
Infarct
papillary
Anterior Septum (6)
Anterior Free Wall (1)
Lateral Wall (2)
Posterior Wall (3)
Inferior Free Wall (4)
Posterior Septal Wall (5)
B
C
%age change in peak strain after 2 weeks pacing
100
75
50
25
0
-25
-50
unpaced
monotonic
RMH
Radial
Circumferential

Figure 4 Cardiac synchrony after 2 weeks of monotonic or RMH pacing

Representative examples of peak strain traces for 6 cardiac segments overlaying M-mode view of parasternal LV long axis are shown and were used to calculate the standard deviation of time-to-peak (TTP) strain as a measure of cardiac dyssynchrony. Examples are taken at the end of 2 weeks unpaced (A), monotonically-paced (B) and RMH-paced (C). The grouped data of the change in dyssynchrony for each treatment is shown (D). The change in myocardial radial and longitudinal peak strain (E) and peak strain rate (F) from the parasternal long axis view are shown for each pacing treatment group.

Data are mean ± SEM.

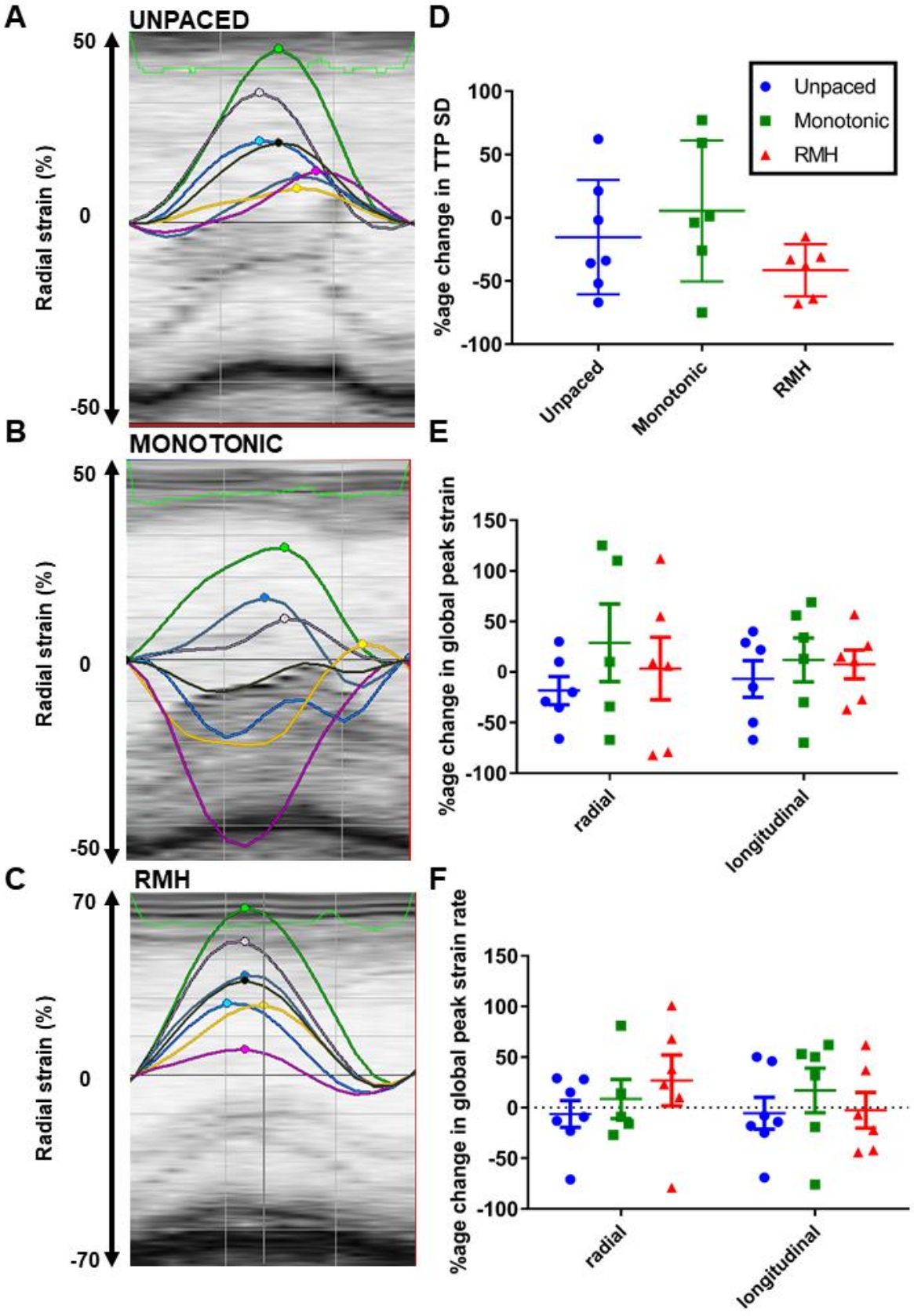

A
UNPACED
50
0
-50
Radial strain (%)
B
MONOTONIC
50
0
-50
Radial strain (%)
C
RMH
70
0
-70
Radial strain (%)
D
Unpaced
Monotonic
RMH
%age change in TTP SD
100
50
0
-50
-100
Unpaced
Monotonic
RMH
E
%age change in global peak strain
150
100
50
0
-50
-100
radial
longitudinal
F
%age change in global peak strain rate
150
100
50
0
-50
-100
radial
longitudinal

Figure 5 Change in diastolic function after 2 weeks of monotonic or RMH pacing

A) Echocardiographic analysis of LV posterior wall thickness (PW) relative to pre-pacing period. B) No significant change in LVPW thickness was observed in any group (One-sample T test compared to null effect). C) Representative example of a transverse cardiac section stained with picrosirius red, viewed under bright light microscopy and a sample location used for collagen quantification inset, viewed under polarised light. D) Collagen infiltration following 2 weeks of pacing was not different between treatment groups.

Data are mean ± SEM. * is $P < 0.05$ for comparisons between treatments (Unpaired t test)

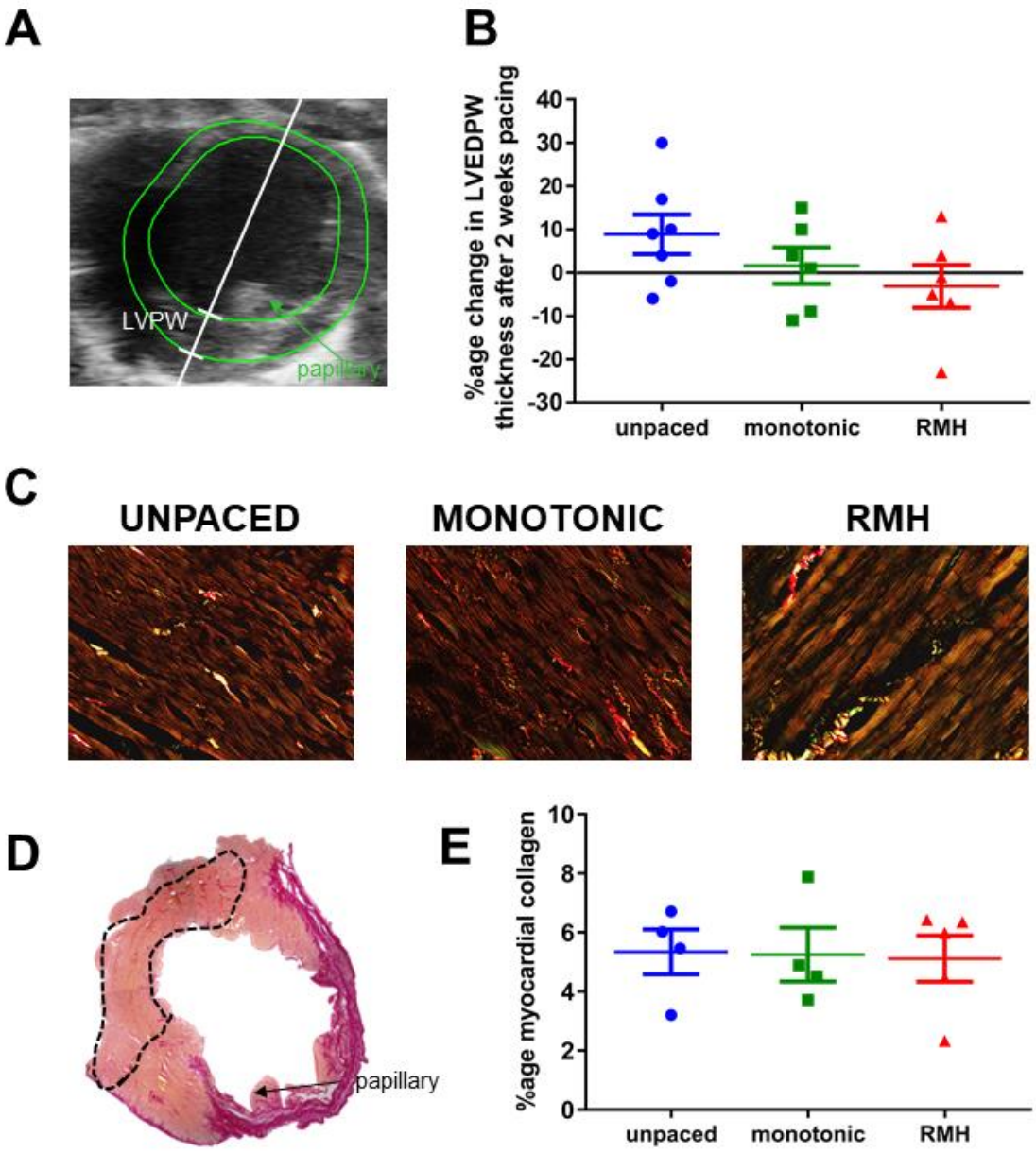
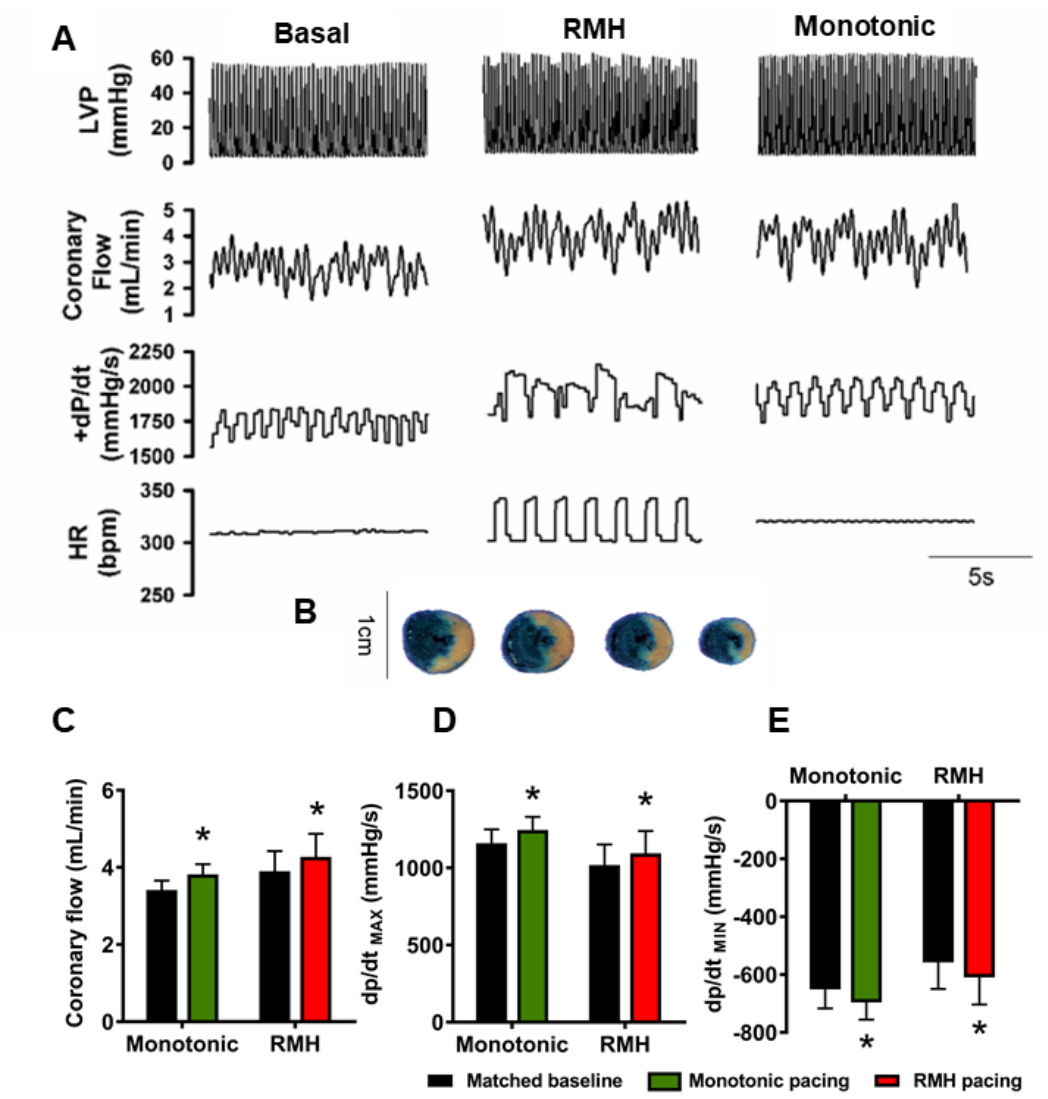

A
LVPW
papillary
B
%age change in LVEDPW thickness after 2 weeks pacing
40
30
20
10
0
-10
-20
-30
unpaced
monotonic
RMH
C
UNPACED
MONOTONIC
RMH
D
papillary
E
%age myocardial collagen
10
8
6
4
2
0
unpaced
monotonic
RMH

Figure 6 The effects of RSA on coronary flow and LV contractility in the isolated perfused heart preparation.

Representative recordings from the Langendorff heart preparation during baseline, simulated RMH and monotonic pacing are shown in A. Heart slices stained with Evans' blue after left coronary artery ligation are shown in B. The heart cross-sections have a non-infarcted blue-marked area contrasting with an infarction area that did not absorb the dye. Simulated RMH and monotonic sinoatrial node pacing increases coronary blood flow (C), LV contractility (+dP/dt) (D), and LV relaxation (E) after MI.

Data are mean ± SEM. * $p<0.05$ compared to basal value within treatment groups (Paired t-test).


A
Basal
RMH
Monotonic
LVP (mmHg)
60
40
20
0
Coronary Flow (mL/min)
5
4
3
2
1
+dP/dt (mmHg/s)
2250
2000
1750
1500
HR (bpm)
350
300
250
5s
B
1cm
C
Coronary flow (mL/min)
6
4
2
0
Monotonic
RMH
D
dp/dt MAX (mmHg/s)
1500
1000
500
0
Monotonic
RMH
E
Monotonic
RMH
dp/dt MIN (mmHg/s)
0
-200
-400
-600
-800
Matched baseline
Monotonic pacing
RMH pacing