Influence of ivabradine on reverse remodelling during mechanical unloading

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Received 22 June 2012; revised 24 September 2012; accepted 12 October 2012; online publish-ahead-of-print 18 October 2012

Aims
Ivabradine (Iva) has shown beneficial structural and functional effects in clinical and experimental heart failure (HF), but its action in combination with mechanical unloading (MU), such as during treatment with left ventricular assist devices (LVAD), is unknown. The aim of this study was to investigate the effects of Iva during MU, in a rodent model of HF.

Methods and results
We studied the chronic effects (4 weeks) of Iva (10 mg/kg/day) alone and in combination with MU [induced by heterotopic abdominal heart transplantation (HATx)] on whole-heart and cellular structure, function, and excitation–contraction (E–C) coupling in a rodent (rat) model of HF, 12 weeks post-left coronary artery (LCA) ligation. Effects of Iva were compared with those of β-blockade using metoprolol [(Met), 250 mg/kg/day]. Iva, but not Met, reversed myocardial fibrosis, alone and in combination with MU. MU-induced restoration of deranged E–C coupling was enhanced by Iva to a greater extent than Met: both Iva and Met enhanced the recovery of the Ca2+ transient amplitude and the sarcoplasmic reticulum (SR) Ca2+ content, but Iva alone maintained MU-induced normalization of L-type Ca2+ current and t-tubule abnormalities. Met prevented MU-induced reduction in the myocardial size (myocardial atrophy); Iva had no effect on this parameter.

Conclusion
Iva shows beneficial structural and E–C coupling effects during MU: Iva reverses myocardial fibrosis and enhances the restoration of deranged E–C coupling, displaying more beneficial effects than that of Met. These results suggest that Iva may prove effective in enhancing functional recovery in heart failure patients receiving LVAD therapy.

Keywords
Heart rate • Heart failure • Cardiovascular surgery • Ventricular assist device • Reverse remodelling

1. Introduction
Left ventricular assist devices (LVADs) are increasingly used in end-stage heart failure (HF) treatment, primarily as bridge to transplantation.1 Mechanical unloading (MU) of the failing LV with LVADs is associated with reverse remodelling of diseased myocardium, and significant beneficial cellular, molecular, electrophysiological, and structural changes.2 Despite these effects, adequate functional recovery permitting device explantation [bridge to recovery (BTR)] remains rare (4–10%).2,3 The reasons for this disparity are unknown, but are likely due to detrimental changes induced by prolonged unloading,4,5 such as myocardial fibrosis,6 deranged excitation–contraction (E–C) coupling,7 and myocardial atrophy.5 Prevention of these effects by pharmacological agents has attracted considerable interest.8–11

Ivabradine (Iva) is a heart rate reduction (HRR) agent that selectively inhibits the pacemaker hyperpolarization-activated If current in the sinoatrial node (SAN), causing a dose-dependent HRR without effects on conduction, inotropy, or blood pressure.12 Iva has demonstrated added clinical benefit when combined with β-blockade in HF therapy,13 showing beneficial LV structural remodelling effects in HF,14 and cardiac transplant patients.15,16 In post-infarction rodent HF, Iva shows multiple beneficial effects: Iva improves whole-heart systolic17–20 and diastolic function,21 decreases interstitial18,19,21 and...
peri-vascular fibrosis, increases angiogenesis, and myocardial perfusion, improves Ca²⁺ handling, and decreases mortality and ventricular excitability.

The effects of Iva during MU are unknown. Iva’s ability to improve cardiac fibrosis, Ca²⁺ handling, and LV structure and function were used for all experiments to avoid the need for immunosuppression. Male syngeneic Lewis rats (10 weeks old, weight ≏ 200 g, Harlan, UK) were used for all experiments to enhance myocardial recovery in patients receiving LVAD support. Therefore, in this study, we tested the hypothesis that Iva would positively enhance reverse remodelling, and promote E–C coupling recovery during MU. We tested this hypothesis by examining the chronic effects of Iva on whole-heart/cell size, function, fibrosis, E–C coupling, and t-tubule structure in a rodent model of HF, and during MU, and compared them to those of β-blockade with Met.

2. Methods

The investigation conformed to the Guide for the care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No.85–23, revised 1996). Experiments were approved by the Harefield Heart Science Centre ethics review panel. (For extended methods, please refer to Supplementary material online).

2.1 Animal models

2.1.1 Induction of heart failure

HF was induced via permanent left coronary artery (LCA) ligation as previously described. Animals were anaesthetized with isoflurane (5% induction, 1.5% maintenance) mixed with O₂ (2–3 L/min), intubated and mechanically ventilated via a rodent ventilator, and a left thoracotomy performed. The pericardium was opened and heart visualized, the proximal LCA was identified and a suture (6–0 prolene) placed underneath the artery and lightly tied. Haemostasis was achieved and the chest closed. Sham-operated animals underwent the identical protocol, but the suture was not tied. The adequacy of anaesthesia was monitored by the loss of reflexes, degree of muscle relaxation, and respiration rate. Respiration rate and body temperature were monitored throughout procedures and body temperature maintained at 37 °C, via heating mat. Acute 24 h mortality was 25% in the LCA ligation group and 0% in the sham group. Male syngeneic Lewis rats (10 weeks old, ~200 g, Harlan, UK) were used for all experiments to avoid the need for immunosuppression following subsequent heterotopic abdominal transplant (HATx) in the MU groups. One week after surgery LV dimensions and function (ejection fraction [EF]) were assessed by transthoracic echocardiography (TTE) (Acuson Sequoia™ 256; Acuson, USA), as previously described. LAD ligation animals with an EF <40% were included in the study, as in our experience animals with baseline function greater than this were unlikely to develop severe dysfunction 12 weeks following surgery. Twelve weeks following LCA ligation the presence of severe cardiac dysfunction was confirmed via TTE, and animals were randomized to groups (n = 8). All groups had similar average EF and variability. 3 month mortality was 1% following LCA ligation and 0% in sham group. TTE was performed under general anaesthesia with isoflurane (5% induction, 1.0% maintenance) mixed with O₂ (2–3 L/min) to provide adequate sedation but minimal cardiac depression.

To test the effects of Iva and Met during MU, failing hearts were transplanted into the abdomen of control syngeneic male Lewis rats (weight ~250 g), as previously described, 12 weeks after LCA ligation, for a period of 4 weeks. Drug doses were selected based on a previous study showing them to cause equal HRR in non-transplanted failing hearts. In brief, both animals (recipient and donor) were anaesthetized with isoflurane (5% induction, 1.0% maintenance) mixed with O₂ (2–3 L/min), and self-ventilated throughout the procedure. The failing heart was removed from the donor animal under cardioplegic arrest, and the donor aorta anastomosed to the recipient abdominal aorta and the donor pulmonary artery to the recipient inferior vena cava. Total ischaemic time was <40 min and operative mortality <4%, with no deaths occurring in any of the treatment groups during the 4-week treatment period. The adequacy of anaesthesia was monitored by the loss of reflexes, degree of muscle relaxation, and respiration rate. Respiration rate and body temperature were monitored throughout procedures and body temperature maintained at 37 °C, via heating mat. Recipients were then assigned to three treatment groups receiving water containing: (i) Iva (Servier, France) 10 mg/kg/day, (ii) Met (Sigma, England) 250 mg/kg/day, and (iii) no treatment (MUHF + Iva, MUHF + Met and MUHF, respectively). Treatment was commenced 12 weeks after LCA ligation, immediately after transplantation, and for the duration of 4 weeks.

The effects of Iva and Met on non-transplanted failing hearts were also studied for comparison. HF animals were randomly allocated to three treatment groups: (i) Iva 10 mg/kg/day in drinking water, (ii) Met 250 mg/kg/day in drinking water, and (iii) no treatment receiving normal drinking water (HF + Iva, HF + Met and HF, respectively). Treatment duration was 4 weeks. At the end of the treatment period change in vivo function was measured via TTE (Supplementary material online, Table S1).

Weekly in vivo assessment of HF in non-anaesthetized animals was performed using implantable ECG telemetry transmitters (CA-F40 Data Sciences International, Minneapolis, MN, USA). Telemetry devices were implanted subcutaneously (SC), under general anaesthesia using isoflurane (5% induction, 1.0% maintenance) mixed with O₂ (2–3 L/min).

2.1.2 Animal anaesthesia, analgesia, and euthanasia

The adequacy of anaesthesia during all procedures was monitored by the loss of reflexes, degree of muscle relaxation, and respiration rate. Respiration rate and body temperature were monitored throughout procedures and body temperature maintained at 37 °C, via heating mat. Analgesia was provided by SC injection of ketamine (ketalar, 10 mg/kg) and xylazine (xylazine hydrochloride, 1 mg/kg) 30 min prior to surgery. LCA ligation and implantation of telemetry device, a single dose of 0.03 mg/kg was utilized, and for HATx both the donor and recipient received a single dose of 0.05 mg/kg. Post-operative condition of the rats was monitored, and repeat SC injection administered as required (0.01–0.05 mg/kg) every 12 h during the first week post-operative. At the end of the 4-week treatment period, animals were sacrificed via scheduled cervical dislocation.

In each group, four hearts were utilized for acute cellular experiments, and four for histological analysis.

2.1.3 Histological analysis of myocardial fibrosis

Interstitial myocardial fibrosis was quantified using a well-established technique. Longitudinal slices were obtained from viable LV free wall, processed and incubated with the collagen specific dye picrosirius red. Stained sections were viewed under bright field and using red fluorescence filter at ×20 magnification on a Zeiss Axioscop microscope with a Nikon D600 camera, and Image fibrosis analysis was carried out using NIS elements 3.0 software (Nikon, UK). The relative area of myocardial interstitial fibrosis was calculated as the area fraction of the visual field stained picrosirius red [collagen area fraction (CAF)]. All morphometric data were collected by the same observer in a blinded fashion. CAF was measured at the end of the treatment period, i.e. 16 weeks after LCA ligation, in all treatment groups. To quantify pre-treatment levels of myocardial fibrosis in failing animals, CAF was also specifically measured in a separate group of HF rats (n = 4), 12 weeks after LCA ligation.

2.1.4 Cardiomyocyte studies

LV cardiomyocytes were isolated by standard enzymatic digestion using collagenase type 2 (1 mg/mL, Worthington) and hyaluronidase (0.6 mg/mL, Sigma) for 8–10 min and used within 6 h.
2.1.4.1 Assessment of sarcomere shortening and Ca$^{2+}$ handling
Simultaneous assessment of sarcomere shortening and Ca$^{2+}$ handling in field stimulated cardiomyocytes was performed using an Ionoptix system (Ionoptix Corporation, USA) and the Ca$^{2+}$ sensitive dye Indo-1 (Indo-1 AM, Molecular Probes). In addition, sarcoplasmic reticulum (SR) Ca$^{2+}$ content, fractional SR Ca$^{2+}$ release (ratio of stimulated Ca$^{2+}$ transient over caffeine-induced Ca$^{2+}$ transient), SR Ca$^{2+}$ uptake, and sodium–calcium exchanger (NCX) contribution to Ca$^{2+}$ extrusion were assessed using rapid caffeine application, as previously described.23

2.1.4.2 Assessment of electrophysiological parameters
Cells were studied using a MultiClamp 700A (Axon Instruments) in whole-cell patch configuration. Current–voltage relationships for L-type Ca$^{2+}$ current were studied and normalized to cell capacitance as previously described.23

2.1.4.3 Assessment of cell volume, t-tubules, and Ca$^{2+}$ sparks with confocal microscopy
Cell volume and t-tubule organization were studied using the membrane binding dye di-B-Anepps (Molecular Probes, Eugene, OR, USA) and the Zeiss Axiowert microscope (Carl Zeiss, Oberkochen, Germany) with an LSM 510 confocal attachment. In addition, Ca$^{2+}$ sparks were assessed using the fluorescent dye fluo-4 AM (Molecular Probes) and the same confocal microscope as previously described.28

2.2 Statistical analysis
Statistical comparison of data was performed using one-way analysis of variance followed by the Bonferroni post hoc test for individual significant differences, or Student’s t-test where appropriate. All statistical analyses were performed using Prism 4 software (Graph-Pad Software, Inc.) and P < 0.05 was considered significant. Data are expressed as mean ± SEM (n), where n is the number of cells unless otherwise specified. All experiments were performed using a minimum of four animals, unless otherwise stated.

3. Results

3.1 Heart rate
Average HR was equal between the sham-operated group and the HF group (P > 0.05, ~330 b.p.m.), and, as expected for the doses employed, Iva and Met therapy caused equal HRR (~20%) in failing hearts, at all-time-points (Figure 1A). MU alone also caused HRR (~20%) at all-time-points (Figure 1B). Iva and Met therapy caused further HRR during MU, but mean HR was lower in Iva-treated (~25% HRR) compared with MU alone, than the Met-treated group during MU (~15% HRR) (Figure 1B and C).

3.2 Myocardial fibrosis
HF was associated with increased myocardial fibrosis (Figure 2). The extent of fibrosis was similar in the HF group and the MUHF group. Iva reduced levels of fibrosis during MU, whereas Met induced a small but significant increase in fibrosis during MU (Figure 2). To determine whether Iva prevents or reverses fibrosis during MU, we compared the extent of fibrosis in Iva-treated animals to those from failing animals 12-week post-LCA ligation, i.e. pre-treatment. The CAF index was lower in MUHF + Iva compared with pre-treatment group, suggesting that effects of Iva were due to reversal, not prevention of fibrosis during MU (Figure 2G). Analysis of Iva-treated non-transplanted failing hearts showed similar results. Iva reversed HF-induced interstitial fibrosis, whereas Met had no effect (Figure 3).

3.3 E–C coupling

3.3.1 Cardiomyocyte contractility
HF did not affect amplitude of sarcomeric contraction, but decreased speed of sarcomeric contraction and relaxation (Figure 4B–D). Contraction amplitude was greater in the MUHF + Iva group than MUHF + Met (Figure 4B). MU alone normalized speed of contraction; the combination with either Iva or Met had no additive effect on this parameter (Table 1, Figure 4C). MU increased speed of relaxation, but this effect was antagonized by both Iva and Met (Table 1, Figure 4D).

3.3.2 Cardiomyocyte Ca$^{2+}$ cycling, L-type Ca$^{2+}$ current, and t-tubule density
HF was associated with deranged Ca$^{2+}$ cycling; Ca$^{2+}$ transient amplitude, speed of Ca$^{2+}$ release, and SR Ca$^{2+}$ content were all decreased (Table 1, Figure 4), whereas speed of Ca$^{2+}$ extrusion and fractional SR Ca$^{2+}$ release were unaffected. MU alone normalized Ca$^{2+}$ transient amplitude, speed of Ca$^{2+}$ release, and SR Ca$^{2+}$ content. Addition of Iva or Met significantly enhanced the recovery of these parameters (Table 1, Figure 4F–H). MU alone had no effect on speed of Ca$^{2+}$ extrusion, but combination with either Iva or Met caused equal slowing of a Ca$^{2+}$ transient decline (Table 1). MU alone did not alter fractional SR Ca$^{2+}$ release, but this parameter was enhanced to supra-sham levels by combination with either Iva or Met (Table 1).

Reduction in the SR Ca$^{2+}$ content can be caused by increased Ca$^{2+}$ leak secondary to altered RyR function.29 Ca$^{2+}$ sparks are a measure of diastolic SR Ca$^{2+}$ release and are complex entities that remain only partially understood.30 Ca$^{2+}$ spark frequency was increased in HF compared with the sham group [(sparks/100 μm²)/s]: sham 0.80 ± 0.17 (37) vs. HF 3.00 ± 0.34 (40); P < 0.001), MU alone normalized this effect in HF myocytes [(sparks/100 μm²)/s]: MUHF 0.82 ± 0.13 (36) vs. HF 3.00 ± 0.34 (40); P < 0.001). Neither drug affected this recovery [(sparks/100 μm²)/s]: MUHF + Iva 1.61 ± 0.37 (24) vs. MUHF + Met (30) 1.70 ± 0.26; P > 0.05, both P > 0.05 vs. MUHF]. MUH induced depression of L-type Ca$^{2+}$ current (Figure 5A) and was associated with reduced t-tubule density (Figure 5B). MU normalized both these parameters, and this effect was maintained by Iva but abolished by Met (Figure 5A and B). A similar effect was observed in non-transplanted failing hearts: Iva normalized L-type Ca$^{2+}$ current and t-tubule density, whereas Met had no effect (Figure 6A and B).

3.4 Myocardial atrophy
LCA ligation produced cardiac hypertrophy with an increase in both heart weight (HW) and cardiomyocyte volume (Figure 7). MU of failing hearts induced a reduction in whole-heart/cell size (myocardial atrophy); both HW and cardiomyocyte volumes falling below sham values (Figure 7). Iva therapy had no effect on MU-induced myocardial atrophy, whereas Met prevented atrophy, with no difference in mean HWs or cardiomyocyte volumes compared with the sham group.

4. Discussion
This study investigated the effects of Iva on three myocardial factors that are likely to oppose functional recovery during MU: fibrosis, derangement of E–C coupling, and atrophy. The β-blocker Met was also studied for comparison. Our results show that Iva reverses HF-induced myocardial fibrosis during MU. In addition, Iva maintains normalization of L-type Ca$^{2+}$ current and t-tubule density, and...
Figure 1  Average HR of Iva-treated and Met-treated failing hearts (A) and transplanted failing hearts (B) measured using telemetry devices. Both treatments reduced HR equally by $\sim$20% at all-time-points in failing hearts. In transplanted hearts, Iva caused slightly greater HRR than Met. ***P < 0.001 vs. HF, ****P < 0.001 vs. MUHF and ***P < 0.01 vs. MUHF + Iva, all groups (n = 4). The percentage decrease in HR caused by Iva and Met is shown (C) Iva caused $\sim$25% and Met $\sim$15% HRR during MU **P < 0.01 and ***P < 0.001.

Figure 2  The effect of Iva and Met on interstitial fibrosis during MU. LV free wall sections from sham (A), HF (B), MUHF (C), Iva-treated (D) and Met-treated (E) animals were stained with picrosirius red. CAF was increased in HF (F). CAF was similar in the MUHF and the HF group, but significantly reduced by Iva (F). CAF in the MUHF + Iva group was lower than in HF animals 12-week post-LAD ligation (pre-treatment), suggesting that Iva reversed fibrosis during unloading (G) **P < 0.01 and ***P < 0.001. Data acquired from a minimum of 60 ventricular sections per group.
results are conflicting; studies reveal both increased 36 – 38 and alters myocardial fibrosis and collagen composition, but quantitative ery during LVAD support,6 with elevated levels of cardiac fibrosis is a proposed therapeutic target in maximizing functional recov-

curred. This has clinical implications in that it suggests that even late values, suggesting that reversal, not prevention, of fibrosis had oc-

erved. Here, we show for the first time that Iva therapy reduced fibrosis during MU and to below pre-treatment effect of Met on fibrosis is unclear, with animal studies showing reduc-

tion of MU, pharmacotherapy, and fibrosis quantification techniques.42 In contrast, Met antagonizes the normalization of L-type Ca2+

content and Ca2+ transient amplitude during MU. 4.1 Myocardial fibrosis

In agreement with previous studies, Iva therapy attenuated HF-induced interstitial fibrosis,18,19 whereas Met showed no effect. Effect of Met on fibrosis is unclear, with animal studies showing reduc-

ation,31–33 and others no effect.34,35 Metoprolol’s anti-fibrotic effects were generally associated with longer treatment durations, and the comparatively short treatment duration used in this study may explain our results. Here, we show for the first time that Iva therapy reduced fibrosis during MU and to below pre-treatment values, suggesting that reversal, not prevention, of fibrosis had occurred. This has clinical implications in that it suggests that even late administration of Iva could be of benefit. Prevention of myocardial fibrosis is a proposed therapeutic target in maximizing functional recovery during LVAD support,6 with elevated levels of cardiac fibrosis correlating with reduced likelihood of recovery.11,36 LVAD therapy alters myocardial fibrosis and collagen composition, but quantitative results are conflicting; studies reveal both increased36–38 and decreased myocardial fibrosis,39,40 and even a biphasic response following prolonged MU, i.e. ECM expansion followed by subsequent re-

gression.31 Such differences are thought to arise due to the marked heterogeneity between studies with regard to HF aetiology, duration of MU, pharmacotherapy, and fibrosis quantification techniques.42

In our study, MU of failing hearts did not significantly alter the CAF index. Interestingly, Iva continued to reverse fibrosis during MU, whereas Met produced a small increase in this parameter. Thus far, the anti-fibrotic effects of Iva were believed to be secondary, at least in part, to direct/indirect amelioration of heightened renin–angiotensin–aldosterone system, as well as adrenergic axis activa-

tion.18,19 However, our results do not support this hypothesis, as, in our experimental setting, the neurohormonal environment is neutral, provided by a healthy recipient animal. This suggests that Iva may be useful in promoting positive ECM remodelling during LVAD therapy, during which normalization of the neurohumoral en-

vironment occurs,4,43 and potentially acts in synergy with proven agents such as ACE-inhibitors.6,10 Ii channels present in ventricular myocytes, known to be mislocalized or up regulated in HF,44 repres-

ent a potential direct route through which Iva may have induced this effect.

4.2 E–C coupling

MU restored deranged E–C coupling, normalizing Ca2+ transient amplitude, speed of Ca2+ release and sarcomeric contraction, SR Ca2+ content, Ca2+ spark frequency, L-type Ca2+ current, and t-tubule density. LVAD therapy is known to increase SR Ca2+ content,45 L-type Ca2+ current fast inactivation, and Ca2+ transient amplitude,45 such changes correlating with improved myocyte contractility and device explantability.46 Mechanisms underlying such actions are unclear, but LVAD therapy is known to enhance SERCA 2A expression47 and stabilize RyR function.48 In this study, Iva enhanced MU-induced recovery of SR Ca2+ content and Ca2+ transient amplitude, parameters predictive of functional recovery,46 and a similar action was seen with Met, suggesting both drugs may be bene-

ficial in enhancing functional recovery during LVAD support. The exact underlying mechanisms require further definition. The precise role of RyR function is unclear as MU-induced normalization of Ca2+ spark frequency was unaffected by either drug, despite an in-

crease in the SR Ca2+ content. Overall, Iva was associated with more favourable effects than Met, showing greater contraction amplitu-

date, along with the maintenance of L-type Ca2+ current and t-tubule
Table 1  Contractility and Ca\(^{2+}\) handling data (16 weeks after LCA ligation)

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<td><strong>1 Hz sarcomere shortenings</strong></td>
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| Amplitude (µm)      | 0.15 ± 0.01 (50) | 0.15 ± 0.005 (35) | 0.14 ± 0.005 (50) | 0.16 ± 0.01 (54) | 0.14 ± 0.01 (47)***
| Time to 90% peak (ms) | 60.2 ± 2.5 (50) | 70.1 ± 2.1 (35)*** | 60.3 ± 1.9 (50)*** | 57.1 ± 2.2 (55)*** | 55.0 ± 1.4 (45)***
| Speed of relaxation (µm s\(^{-1}\)) | 19.8 ± 2.0 (48) | 13.4 ± 2.4 (34)*** | 15.9 ± 1.6 (50)*** | 12.3 ± 1.8 (53)*** | 13.6 ± 1.9 (45)***
| **1 Hz Ca\(^{2+}\) transient** |          |          |          |            |            |
| Amplitude (ratio units) | 0.17 ± 0.01 (45) | 0.14 ± 0.01 (35)*** | 0.18 ± 0.005 (45)*** | 0.24 ± 0.01 (50)*** | 0.23 ± 0.01 (40)***
| Time to 90% peak (ms) | 18.0 ± 1.1 (50) | 24.3 ± 1.4 (35)*** | 17.8 ± 0.8 (50)*** | 15.6 ± 0.6 (50)*** | 17.1 ± 0.6 (40)***
| Speed of Ca\(^{2+}\) extrusion (ratio unit\(^{-1}\)) | 12.3 ± 0.4 (50) | 13.1 ± 0.3 (35) | 11.7 ± 0.4 (50) | 10.6 ± 0.3 (50)*** | 10.3 ± 0.4 (40)***
| **Caffeine-induced Ca\(^{2+}\) transient** |          |          |          |            |            |
| SR Ca\(^{2+}\) content (ratio units) | 0.21 ± 0.01 (40) | 0.16 ± 0.01 (28)*** | 0.23 ± 0.01 (40)*** | 0.28 ± 0.01 (45)*** | 0.30 ± 0.01 (40)***
| Fractional release of SR (%) | 81.4 ± 2.1 (40) | 80.5 ± 3.4 (28) | 84.5 ± 2.2 (40) | 88.5 ± 3.1 (40)*** | 87.1 ± 2.1 (35)***
| Contribution to Ca\(^{2+}\) decay (%) | NCX 7.1 ± 0.6 (28) | 7.6 ± 0.7 (40) | 8.5 ± 0.6 (45)*** | 9.9 ± 0.5 (45)*** | 9.8 ± 0.5 (28)***
| SERCA 92.9 ± 1.1 (28) | 92.4 ± 0.7 (40) | 91.5 ± 0.4 (45)*** | 90.1 ± 0.4 (45)*** | 90.2 ± 1.3 (30)*** |

\( ^{a}p < 0.05 \),  \( ^{b}p < 0.01 \),  \( ^{***}p < 0.001 \) vs. sham.

\( ^{\dagger}p < 0.05 \),  \( ^{\ddagger}p < 0.01 \),  \( ^{\ast\ast\ast}p < 0.001 \) vs. HF.

\( ^{\wedge}p < 0.05 \),  \( ^{\wedge\wedge}p < 0.01 \),  \( ^{\ast\ast\ast\ast}p < 0.001 \) vs. HF.

\( ^{\dagger\dagger}p < 0.001 \) vs. MUHF.

\( ^{\ddagger\ddagger}p < 0.001 \) vs. MUHF + Iva.
**Figure 5** MU-induced normalization of depressed L-type Ca\(^{2+}\) current (A) and t-tubule density (measured by % Di-8-Anepps-stained area) (B). Iva did not alter these effects, whereas Met abolished them. *P < 0.05, **P < 0.01, and ***P < 0.001. Representative Di-8-Anepps-stained cells from sham (C), HF (D), MUHF (E), MUHF + Iva (F), and MUHF + Met (G) groups.

**Figure 6** HF-induced depression of L-type Ca\(^{2+}\) current (A) and t-tubule density (measured by % Di-8-Anepps-stained area) (B). Iva alone normalized these parameters, whereas Met had no effect. ***P < 0.001. Representative Di-8-Anepps-stained cells from sham (C), HF (D), HF + Iva (E), and HF + Met (F) groups.
density recovery, T-tubule disruption uncouples the normally tight relationship between L-type Ca\(^{2+}\) channels and RyRs, reducing E–C coupling efficiency.\(^30\) Iva therapy, but not Met, normalized t-tubule density and L-type Ca\(^{2+}\) current in non-transplanted failing hearts, possibly improving such coupling, and represents a novel action, that may have contributed to the improved whole-heart contractile function demonstrated in our, as well as previous rodent HF studies.\(^17\) – 20 Despite several beneficial effects during MU, the negative lusitropy and slowing of Ca\(^{2+}\) extrusion during MU highlight potentially unwanted effects of Iva and Met that require further investigation.

### 4.3 Myocardial atrophy

LVAD therapy causes a regression of cardiac and myocyte hypertrophy.\(^49\) The extent of this regression correlates with the duration of support.\(^39\) Whether prolonged clinical LVAD support brings about myocyte atrophy, the decrease in the myocyte size to subnormal values (not necessarily dysfunctional), is unclear and remains controversial.\(^50\)

Along with myocardial fibrosis, prevention of myocardial atrophy during prolonged LVAD therapy is a proposed therapeutic target for enhancing BTR.\(^5,24\) but the precise importance of size in relation to function remains unproven. Clenbuterol, in combination with aggressive medical therapy, has enhanced BTR rates as part of the Harefield protocol,\(^8\) but evidence supporting an anti-atrophic action is minimal.\(^46\) Experimentally, HATx has been convincingly demonstrated to induce myocardial atrophy.\(^24,25,51\) We show that Iva did not prevent MU-induced atrophy, but surprisingly Met proved successful. Hitherto, no pharmacological agent has proved effective in attenuating myocardial atrophy, with clenbuterol only limiting regression of hypertrophy of failing rat hearts, after a short 1-week period of MU,\(^23\) when atrophic remodelling is submaximal.\(^24,25\) Partial haemodynamic loading\(^51,52\) and reloading\(^53\) of unloaded rodent LVs attenuates atrophy, re-iterating the importance of load in regulating cardiac mass.\(^25,51\) Such loading, potentially brought about by HRR and subsequent augmentation of LV filling, may have favoured anti-atrophic actions of Met, but the lack of the effect of Iva, despite a slightly larger HRR, makes this mechanism unlikely.

### 4.4 In vivo whole-heart function and heart rate

Echocardiographic assessment of non-transplanted failing hearts confirmed that Iva significantly improves LVEF and LVFS in HF as previously shown,\(^17\) – 20 with Met showing a minimal effect.\(^54\) (Supplementary material online, Table S1). The measurement of in vivo whole-heart contractile function in abdominally transplanted hearts is problematic, and the reliability and value of standard echocardiography, MRI, and pressure–volume-derived functional parameters are questionable.\(^55\) As such, in vivo whole-heart function was not assessed in this study, and in the view of the distinct treatment effects on
myocardial atrophy and fibrosis would have provided additional valuable information.

The isolated HR effects of MU remain unstudied in both the experimental, and clinical setting. In this study, transplanted HR was lower than non-transplanted, most likely due to cardiac denervation, absence of heightened sympathetico-adrenergic stimulation, and reduced stretch-induced modulation of SAN automaticity.56 HR is a critical regulator of myocardial oxygen delivery and consumption. HRR prolongs diastolic duration, improves coronary flow, and enhances myocardial energetic balance, while concurrently reducing myocardial oxygen consumption.57,58 Therefore, it is reasonable to believe MU-induced HRR contributed to myocyte recovery. In non-transplanted failing hearts, HRR via Iva is associated with improved myocardial atrophy was unaffected by Iva but was prevented by Met. These results suggest that Iva may prevent LVAD-induced negative remodelling of the ECM and E–C coupling, and therefore in enhancing functional myocardial recovery in HF patients receiving LVAD support.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Acknowledgements**

We extend our thanks to Muriel Bouly and Jerome Roussel, for critical comments on this manuscript and Servier, France for providing us with the ivabradine in this study.

**Conflict of interest:** none declared.

**Funding**

We are grateful to the Magdi Yacoub Institute (HSC 87/04) for financial support.

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Ivabradine and mechanical unloading


