British Journal of Pharmacology

DOI:10.1111/bph.13332
www.brjpharmacol.org

RESEARCH PAPER

Contractile function assessment by intraventricular balloon alters the ability of regional ischaemia to evoke ventricular fibrillation

Catherine D E Wilder, Radwa Masoud, Duygu Yazar, Brett A O'Brien, Thomas R Eykyn and Michael J Curtis

Cardiovascular Division, King's College London, London, UK

Correspondence
Michael J Curtis, Cardiovascular Division, Faculty of Life Sciences and Medicine, The Rayne Institute, St Thomas' Hospital, London SE17EH, UK.
E-mail: michael.curtis@kcl.ac.uk

Received
4 June 2015
Revised
4 September 2015
Accepted
10 September 2015

BACKGROUND AND PURPOSE

In drug research using the rat Langendorff heart preparation, it is possible to study left ventricular (LV) *contractility* using an intraventricular balloon (IVB), and *arrhythmogenesis* during coronary ligation-induced regional ischaemia. Assessing *both concurrently* would halve animal requirements. We aimed to test the validity of this approach.

EXPERIMENTAL APPROACH

The electrocardiogram (ECG) and LV function (IVB) were recorded during regional ischaemia of different extents in a randomized and blinded study.

KEY RESULTS

IVB-induced proarrhythmia was anticipated, but in hearts with an ischaemic zone (IZ) made deliberately small, an inflated IVB reduced ischaemia-induced ventricular fibrillation (VF) incidence as a trend. Repeating studies in hearts with large IZs revealed the effect to be significant. There were no changes in QT interval or other variables that might explain the effect. Insertion of an IVB that was minimally inflated had no effect on any variable compared with 'no IVB' controls. The antiarrhythmic effect of verapamil (a positive control drug) was unaffected by IVB inflation. Removal of an inflated (but not a non-inflated) IVB caused a release of lactate commensurate with reperfusion of an endocardial/subendocardial layer of IVB-induced ischaemia. This was confirmed by intracellular ^{31}phosphorus (^{31}P) nuclear magnetic resonance (NMR) spectroscopy.

CONCLUSIONS AND IMPLICATIONS

IVB inflation does not inhibit VF suppression by a standard drug, but it has profound antiarrhythmic effects of its own, likely to be due to inflation-induced localized ischaemia. This means rhythm and contractility cannot be assessed concurrently by this approach, with implications for drug discovery and safety assessment.

Abbreviations

BG, bigeminy; IVB, intraventricular balloon; IZ, ischaemic zone; LV, left ventricular; NMR, nuclear magnetic resonance; NO, nitric oxide; PCr, phosphocreatine; P_i, inorganic phosphate; TVW, total ventricular weight; UZ, uninvolved zone; VF, ventricular fibrillation; VPB, ventricular premature beat; VT, ventricular tachycardia

Tables of Links

TARGETS
Ion channels
$Ca_v1.2$ channels

LIGANDS
Verapamil

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

The present study reports a refinement of a common model for studying drug actions on the heart, identifies an unexpected alteration in the model's bioassay characteristics, explains this mechanistically and provides guidance on future usage.

An intraventricular balloon (IVB) is commonly used in the rat isolated Langendorff perfused heart in order to measure left ventricular (LV) contractile function (Ridley and Curtis, 1992; Farkas *et al.*, 1999; Crook and Curtis, 2012). In addition, the rat isolated heart is also used to evaluate ischaemia-induced and reperfusion-induced cardiac arrhythmias such as ventricular fibrillation (VF) (Ridley *et al.*, 1992; Clements-Jewery *et al.*, 2006; Crook and Curtis, 2012). Assessment of rhythm *and* LV contractility simultaneously in a single experiment is a strategy that would give a more comprehensive assessment of drug effects with reduced animal usage. Indeed, the growing interest in the rat Langendorff preparation with an IVB for cardiac safety assessment (Henderson *et al.*, 2013) coupled with its longstanding value for examining drug effects on regional ischaemia-induced arrhythmias (Curtis *et al.*, 2013) indicates that combining assessment into a single experiment is a logical next step.

It is not known whether it is feasible to do this, and there are reasons to suspect that IVB assessment of function may affect arrhythmogenesis owing to effects of LV stretch. Studies on electrically induced arrhythmias with and without ischaemia suggest an increase in VF susceptibility and complexity with LV stretch (Coronel *et al.*, 2002; Parker *et al.*, 2004; Barrabés *et al.*, 2013), and importantly, from a pharmacological perspective, possible alteration of the effects of antiarrhythmic drugs (Chorro *et al.*, 2000). However, the effects of LV stretch on VF induced by ischaemia (rather than by electrical stimulation) have not been systematically established, especially in the versatile rat Langendorff preparation.

In rat isolated hearts subjected to regional ischaemia, the incidence of VF is dependent on the size of the ischaemic territory (Ridley *et al.*, 1992) as it is in larger species (Austin *et al.*, 1982). Moreover, in the rat isolated heart, the size of the territory, that is, the ischaemic zone (IZ), can be varied easily by selecting the position of the coronary ligature (Ridley *et al.*, 1992). Proximal ligation of the left coronary artery in rat isolated hearts results in approximately 80% of hearts developing VF, whereas placing the ligature more distally to the atrial appendage reduces VF incidence proportionately (Ridley *et al.*, 1992). This allows evaluation of an alteration in the relationship between VF and IZ by drugs, or by other manipulation such as IVB inflation.

In order to determine the feasibility of combining IVB assessment of contractility with assessment of ischaemia-induced arrhythmias, and assess whether an IVB facilitates ischaemia-induced arrhythmias in the rat isolated heart, we deliberately made the IZ smaller (~35% mean of the ventricular mass, with individual values <40%) than is typically used for antiarrhythmic drug studies, thereby generating a more moderate baseline incidence of VF (~33%) to permit detection of any increase in VF incidence. In separate studies, we examined whether IVB inflation affected the antiarrhythmic action of verapamil, a drug well characterized in the unloaded preparation (Farkas *et al.*, 1999) and which has been reported to alter the pattern of VF in rabbit hearts subjected to IVB inflation (Chorro *et al.*, 2000). Coronary effluent lactate release and ^{31}P nuclear magnetic resonance (NMR) spectroscopy (providing data on intracellular pH, ATP and other variables susceptible to ischaemia) were analysed in two separate studies in order to explore whether IVB-induced myocardial injury was the mechanism that determined outcome.

Methods

Concordance with ARRIVE, ethical and legal requirements and new guidance on design and analysis

Animal housing and husbandry were exactly as previously described (Andrag and Curtis, 2013), in full compliance with ARRIVE (Kilkenny *et al.*, 2010) and United Kingdom Home Office *Guide on the Operation of the Animals (Scientific Procedures) Act 1986*, and the new guidance on design and analysis for British Journal of Pharmacology (Curtis *et al.*, 2015) with blinding and randomization throughout, and are therefore not described in detail here. A total of 164 animals were used in the experiments described here.

Animals and general experimental methods

Rats (male Wistar; Harlan UK; 280–400 g) were anaesthetized with a lethal dose of sodium pentobarbitone (170 mg kg^{-1} i.p.) and given sodium heparin (160 IU kg^{-1} i.p.) in order to preclude blood clot formation within the coronary vasculature. The high dose of pentobarbitone obtained a rapid onset

of surgical level anaesthesia, determined by the absence of pedal and corneal reflexes whereupon hearts were immediately excised (causing death by exsanguination) and arrested in ice-cold modified Krebs solution, containing NaCl 118.5 mM, $CaCl_2$ 1.4 mM, glucose 11.1 mM, $NaHCO_3$ 25.0 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.2 mM and KCl 3 mM. The aorta was cannulated for Langendorff perfusion with a similar solution, gassed and warmed (95% O_2: 5% CO_2; pH 7.4; 37°C). All solutions were filtered (5 μm pore size) before use and delivered at a constant pressure of approximately 80 mmHg. A unipolar electrocardiogram (ECG) recording was used for assessment of cardiac rhythm as previously described (Dhanjal *et al.*, 2013). Small variations in this process were used for ^{31}P NMR spectroscopy studies as detailed in the following text.

To achieve coronary occlusion, a silk suture was placed loosely around the left main coronary artery. Both ends of the suture were threaded through a polythene tube, which was tightened to induce regional ischaemia and later loosened to achieve reperfusion. After reperfusion, the IZ was delineated by re-occlusion during perfusion with disulphine blue dye (Patent blue VF sodium salt, Sigma-Aldrich®, UK) and expressed as a percentage of total ventricular weight (TVW). In initial studies, to generate small IZs (<40% of TVW; $n = 12$ per group), the suture was positioned midway down the coronary tree, towards the apex of the heart, medial to the atrial appendage. In later studies, to give a larger mean IZ (of approximately 50% TVW), the suture was inserted beneath the left atrial appendage, exiting approximately 1 mm from the perimeter of the conus arteriosus.

A deflated compliant non-elastic IVB (Curtis *et al.*, 1986) (Figure 1) attached to a pressure transducer was inserted into the left ventricle via the left atrium to measure contractile function. Diastolic function is denoted by diastolic pressure, and inotropy (force development) is denoted by developed pressure (the difference between peak systolic pressure and minimum diastolic pressure during a single cardiac cycle during sinus rhythm, at least five beats after the cessation of any arrhythmia). To generate a physiological level of LV stretch, typical for studies focused solely on rat LV contractile function (Curtis *et al.*, 1993), the IVB was inflated to achieve a submaximal LV developed pressure (approximately 70% of maximum) that nevertheless exceeded 100 mmHg. To obtain this, we initially added 0.12 mL of saline to the IVB. The choice of 0.12 mL was based on previous experience for hearts of the size chosen, and small adjustments (±0.02 mL) were made if LV developed pressure did not attain 100 mmHg, or if diastolic pressure exceeded 5 mmHg. To control for physical effects of the presence of an IVB itself on rhythm, a separate group of hearts received an IVB with minimal inflation (<0.01 mL). The small volume was added so a pressure could *just* be measured (impossible if none were added). This gave an LV developed pressure at baseline of ~30 mmHg. A third group of hearts with no IVB was included in the study. Hereafter, we refer to the three groups as 'IVB inflated' (~0.12 mL), 'IVB' (<0.01 mL) and 'no IVB' respectively. The IVB technique, together with some caveats concerning IVB manufacture and troubleshooting, is discussed in Clements-Jewery and Curtis (2014).

Figure 1
One of the IVBs used in the present study shown uninflated (for insertion into the left ventricle), minimally inflated (0.01 mL), and inflated (0.12 mL) according to the definitions provided in the text.

Experimental protocols

The study was divided into a sequence of experiments designed to address separate questions. Within each experiment, hearts were randomized to groups, and analysis of variables was undertaken blind. After 5 min of perfusion, all hearts were subjected to regional ischaemia for 120 min (initial set of experiments) or 30 min followed by reperfusion. The 120 min duration was chosen to capture the full spectrum of phase 1 ischaemia-induced ventricular arrhythmias in rat isolated hearts (Ravingerova *et al.*, 1995). The 30 min duration was selected in order to explore the mechanism of IVB effects during the peak of ischaemia-induced phase 1 arrhythmia susceptibility (Clements-Jewery *et al.*, 2002).

In the first experiment, no IVB, IVB and IVB inflated groups with small ($n = 12$ per group) and large IZs ($n = 12$ per group) were compared. In the second study, no IVB, IVB and IVB inflated groups with large IZs ($n = 12$ per group) were perfused with Krebs for 5 min followed by a switch to 0.6 μM verapamil [shown previously to block ischaemia-induced VF in the rat isolated heart with no IVB (Farkas *et al.*, 1999)] for the remainder of the protocol.

In a third study ($n = 5$ per group), in hearts with large IZs, coronary effluent lactate release was measured electrochemically using the 2300 STAT Plus™ lactate analyser (YSI Ltd, UK) to assess whether IVB inflation inadvertently causes endo/subendocardial ischaemia. Baseline lactate turnover and lactate release caused by reperfusion following regional ischaemia were used to benchmark the apparent extent of ischaemia-(and reperfusion-)induced injury associated with IVB inflation (and deflation). After 5 min of baseline recordings, hearts were made regionally ischaemic for 30 min followed by 3 min of reperfusion (sufficient duration to capture the window of lactate release during reperfusion; Apstein *et al.*, 1977; Ceconi *et al.*, 1988; Cargnoni *et al.*, 1996; Ferrari *et al.*, 1996). At 24.30 (min.s) of ischaemia, the IVB was fully and rapidly deflated and removed. Coronary effluent samples were collected at regular and sequential 15 s intervals as outlined in Table 1. Samples were weighed, aliquoted and frozen in liquid nitrogen upon completion of the protocol.

Lactate analysis

Frozen coronary effluent samples were thawed and analysed for lactate content using the 2300 STAT Plus lactate analyser (YSI Ltd, UK). The lactate analyser was calibrated before every sampling. Triplicates were used to determine one accurate value per sample for subsequent determination of group mean values. To unclutter the data presentation, trios of

Table 1

Coronary effluent collection protocol for lactate measurement experiments

	Before ligation		Ischaemia: With balloon[a]	Ischaemia: With balloon[a]	Ischaemia: Balloon removed[a]			Reperfusion		
Time (min.s)	−1.00		+5.00[b]	+20.00	+24.30	+25.15		+29.58	+31.00	+32.00
	−0.45	LIGATION 0.00	+5.15	+20.25	+24.45	+25.30	REPERFUSION 29.58	+30.15	+31.15	+32.15
	−0.30		+5.30	+20.30	+25.00	+25.45		+30.30	+31.30	+32.30
								+30.45	+31.45	+32.45

[a]Only in IVB and IVB inflated groups (no balloon in control group). There were $n = 5$ hearts in each group.
[b]Only seven hearts ($n = 2$ or 3 per group) had measurements taken from 5–5.30 owing to a temporary design oversight.

sequential 15 s samples were combined to give lactate release data in 45 s time bins.

Coronary flow, ECG and pressure trace analysis

Coronary flow was determined by timed collection of coronary effluent (which was weighed; 1 g = 1 mL). Coronary flow was expressed as mL min^{-1} g^{-1} to control for any variations in heart weight. ECG and pressure traces were recorded using PowerLab™ (ADInstruments, UK; sampling rate 4 kHz). Ventricular premature beats (VPBs), bigeminy (BG), salvos, ventricular tachycardia (VT) and VF were defined according to the Lambeth Conventions II (Curtis *et al.*, 2013). Arrhythmias were recorded as either occurring or not occurring in each sequential 5 min interval during the first 30 min of ischaemia, and in subsequent 30 min intervals until the end of ischaemia at 120 min. An arrhythmia score (Table 2) was also assigned to summarize arrhythmia severity and enable parametric statistical tests to be conducted (Curtis and Walker, 1988). Sections of ECG and pressure traces in sinus rhythm were used to obtain heart rate, PR and QT_{90} (QT interval at 90% repolarisation) intervals, and LV diastolic and developed pressures, respectively, using on-screen cursors.

Table 2

Arrhythmia score: quantification of arrhythmia severity

Arrhythmia score	Arrhythmia
0	VPBs in <1 time interval
1	VPBs in >1 time interval
2	BG or salvos
3	VT
4	non-sustained VF (<120 s)
5	sustained VF (>120 s)

BG, bigeminy; VF, ventricular fibrillation; VPB, ventricular premature beat; VT, ventricular tachycardia.

Rationale for selection of verapamil concentration

The concentration of verapamil used was based on previous data by Farkas *et al.* (1999) that showed verapamil at 600 nM was sufficient to abolish ischaemia-induced VF in the rat isolated heart.

^{31}P NMR spectroscopy

For these studies, the perfusion solution was identical except that it was phosphate-free [this modification is necessary to permit measurement of the inorganic phosphate (P_i) peaks with ^{31}P NMR spectroscopy and does not affect haemodynamics or susceptibility to arrhythmogenic effects of ischaemia] (Curtis, 1991). Because the set-up precludes gravity-driven perfusion, a constant pressure of 74 mmHg was maintained with a pump controller feedback system (AD Instruments, Oxford, UK). The delivery tubing was jacketed by circulating warm water such that the perfusion solution at the aortic cannula was maintained at 37°C. Three groups of $n = 5$ (no IVB, IVB and IVB inflated), each with placement of a loose coronary ligature, were prepared as described above. The perfused heart was then placed into an NMR tube (OD 15 mm) (Wilmad, UK) with a sipper line for removal of coronary effluent, and centred within an RF $^{1}H/^{31}P$ dual tune coil within a microimaging probe. A Bruker Avance III 400 MHz wide-bore spectrometer (Bruker, Karlsruhe, Germany) with triple-axis gradients maintained at~313 K using warm water circulation was used for all experiments. Coronary flow and perfusion pressure were monitored throughout perfusion using LabChart software v. 7 (AD Instruments, Australia). ^{31}P spectra were acquired during 10 min of baseline perfusion. The heart was then removed from the NMR apparatus while perfusion was maintained to allow the coronary ligature to be tightened as described above. The heart was immediately returned to the NMR apparatus for a further 30 min. Fully relaxed ^{31}P spectra were acquired with a 60° flip angle, 64 scans, 4 s repetition time, 16 000 data points and 50 p.p.m. sweep width at the following time points relative to the moment of coronary occlusion: −10 to −6 min, −6 to −2 min, 5 to 9 min, 9 to 13 min, 13 to 17 min, 17 to 21 min, 21 to 25 min and 25 to 29 min. Total ventricular tissue, IZ and uninvolved zone (UZ) were weighed at the end of perfusion. NMR data were Fourier transformed with an exponential line

broadening factor of 15 Hz followed by peak phasing and baseline correction. Peaks of interest [phosphocreatine (PCr), P_i in both the IZ and UZ, and β-ATP] in all samples were manually integrated on the same scale, calibrated to the first PCr peak (which was given a value of '1') of the first spectrum of the first heart and then normalized to TVW [with the exception of P_i in the IZ, which was normalized to IZ weight, and P_i in the UZ, which was normalized to TVW (pre-ischaemia) and UZ weight (during ischaemia)]. Intracellular pH values of the IZ (down field P_i peak) and UZ (up field P_i peak) were calculated using the equation $pH = 6.72 + \log_{10}[(\delta ppm - 3.17)/(5.72 - \delta ppm)]$ where 'δppm' is the chemical shift difference between the P_i and PCr peaks (Sidell *et al.*, 2002).

Exclusion criteria

According to published exclusion criteria (Clements-Jewery *et al.*, 2002; Dhanjal *et al.*, 2013), any heart with a sinus rate of less than 200 beats min^{-1} or coronary flow outside the range of 5 to 18 mL min^{-1} g^{-1} 1 min prior to ischaemia, or an IZ <45% unless VF occurred (large IZs) or >40% (small IZs) was excluded from the data set and replaced in order to maintain equal group sizes. Hearts that fell outside these criteria were not excluded if doing so would make no difference to the outcome of the study (statistical analysis of VF incidence data), minimizing needless animal usage.

Hearts were excluded from the lactate analysis or ^{31}P NMR spectroscopy experiments and replaced if there was no instantaneous reduction in coronary flow after ligation, and if there was no increase in flow or lactate release during reperfusion (indicative of poor occlusion, i.e. ligature not tightened effectively); when a heart required to be excluded, typically all three of these criteria were violated.

Data analysis

Gaussian distributed variables were subjected to analysis of variance (ANOVA; 1 way or 2 way, repeated measures) followed by Dunnett's, Tukey's or Sidak's *post hoc* tests if *F* was significant and there was no variance inhomogeneity in accordance with journal guidance (Curtis *et al.*, 2015), with values expressed as mean±SEM. Binomially distributed variables were compared using Fisher's exact tests. $P < 0.05$ was defined as statistically significant.

Materials

We obtained patent blue VF sodium salt and (±)-verapamil hydrochloride from Sigma-Aldrich, UK, and Krebs perfusate salts from VWR International, UK or Fisher Scientific, UK. All test solution stocks and perfusion solutions used water from a PURELAB flex dispenser (ELGA Process Water, UK), resistivity 18 MΩ.

Results

Verification of experimental condition: IZ size

The size of the IZs did not differ between no IVB, IVB and IVB inflated groups within each of the four studies (Figure 2A). IZ sizes differed significantly when this was intended (i.e. large versus small IZ designation) (Figure 2A).

Verification of experimental condition: developed pressure

Inflation of the IVB significantly increased LV developed pressure at baseline versus IVB hearts, as intended (Figure 2B).

Arrhythmias

In hearts with small IZs, IVB inflation was anticipated to increase the incidence of VF during the first 30 min of ischaemia (phase 1 VF; Figure 3E). Instead, it *reduced* the incidence of VF (as a non-significant trend). To test whether the effect was robust, the study was repeated in hearts with large IZs (more scope to detect VF suppression) and a substantial and significant effect was detected (Figure 4E). IVB inflation also significantly reduced the incidence of VT (30–60 min ischaemia; Figure 4D) and a five-point arrhythmia score (Figure 4F) in these hearts.

Nevertheless, in hearts with small IZs, the IVB and IVB inflation significantly *increased* the incidence of less severe phase 1A (0–10 min; Figure 5A) and early phase 2 arrhythmias (>30 min; Figure 3B–D), and a five-point arrhythmia score (Figure 3F + 5B). There was also a non-significant trend to an increase in susceptibility to phase 1A arrhythmias in hearts with large IZs, in which there is less scope to detect an increase owing to the high baseline susceptibility (data not shown).

In verapamil-perfused hearts arrhythmia occurrence ranged from ubiquitous to absent, depending on arrhythmia type (Figure 6), with prevalence inversely related to severity (almost all hearts had VPBs, and none had VF). Thus, the ability of verapamil to abolish the most severe arrhythmias was not affected by an IVB or its inflation. In contrast to its effects in verapamil-free hearts (Figure 4), IVB inflation did not facilitate 1A ischaemic arrhythmias (0–10 min; data not shown).

Haemodynamic and ECG changes

Ischaemia caused polyphasic changes in heart rate, coronary flow and QT typical for the preparation (Clements-Jewery *et al.*, 2002; Farkas and Curtis, 2003; Baker *et al.*, 2006; Dhanjal *et al.*, 2013) which, as expected, were more pronounced in hearts with large IZs versus hearts with small IZs (Figures 7A, B and D and 8A, B and D). However, aside from an IVB-induced QT prolongation in hearts with small IZs (Figure 7D), which was not seen in hearts with large IZs (Figure 8D), the presence or inflation of an IVB had no notable effect on haemodynamic or ECG variables (Figures 7A–D and 8A–D).

The haemodynamic and ECG changes that occurred in verapamil-perfused hearts were similar to those seen previously in hearts without an IVB (Farkas *et al.*, 1999). The presence and inflation of the IVB had no effect on the haemodynamic and ECG effects of verapamil (data not shown).

LV pressure

Ischaemia significantly increased diastolic pressure in IVB inflated hearts, and this was further increased by reperfusion, with effects greater in hearts with larger IZs (Figure 9A and C). Likewise, coronary ligation reduced developed pressure with effects more pronounced in hearts with larger IZs, and more evident in hearts with the IVB inflated (Figure 9B and D).

Verapamil increased diastolic pressure significantly, and this effect was sustained throughout ischaemia and further increased by reperfusion (data not shown). Likewise,

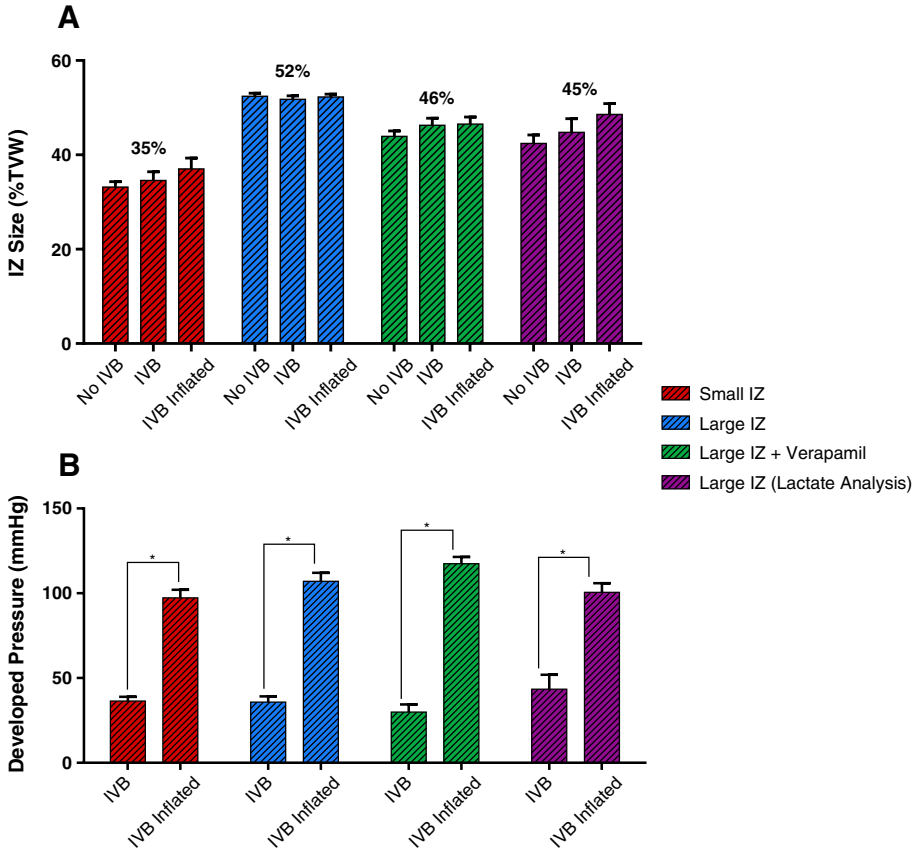

Figure 2

Part A shows IZ sizes from the separate studies. Data expressed as mean ± SEM of group mean % values. There were no significant differences within each separate trio of values. Part B shows LV developed pressure at baseline (pre-drug values). Data expressed as mean ± SEM. All groups are $n = 12$ hearts, except the two 'Large IZ (Lactate Analysis)' groups, which are both $n = 5$, $^*P < 0.05$.

developed pressure was significantly decreased by more than 50% by verapamil (data not shown).

Lactate analysis

Prior to ischaemia, there were no differences in lactate release between groups (Figure 10) indicating that although inflation of the IVB increased developed pressure (Figure 9B), this did not compromise myocardial metabolism and that IVB inflation-induced inotropy does not cause an increase in lactate release. However, there was a significant transient increase in lactate release following IVB deflation and removal (Figure 10). During reperfusion, there was a substantially greater increase in lactate release. This was complete within 2 min of the onset of reperfusion. The total increase in lactate release during this 2 min was unrelated to the absence or earlier presence or inflation of an IVB (note that the IVBs had been removed 5 min before reperfusion), although the increase peaked sooner in hearts that had previously contained an inflated IVB (Figure 10).

^{31}P NMR spectroscopy

Figure 11 shows data from the ^{31}P NMR experiment. Part A shows that ischaemia causes a single P_i peak to become double, indicating the presence of two regions of intracellular pH (regional ischaemia). Part B shows that total PCr values fall dramatically and quickly during ischaemia in all three groups with no discernible difference between them. Part C shows that β-ATP levels fall more gradually during ischaemia, reaching a trough before 20 min of ischaemia in each group, with no differences between groups. Intracellular pH was constant in the UZ but fell quickly during ischaemia in the IZ (Figure 11D). Part E shows that P_i in the IZ rose swiftly and to a similar peak, reached before 20 min of ischaemia, in each group with no discernible differences between groups. However, part F of Figure 11 shows that there was also an increase in P_i in the UZ (that occurred more slowly and to a lower maximum than the increase in the IZ). Moreover, P_i in the UZ 25 min after the start of ischaemia was significantly greater in the IVB inflated hearts versus the no IVB hearts.

Discussion

Overview

Our purpose was to test whether IVB recording of LV pressure in regional ischaemia-induced arrhythmia studies could be used to add data and potentially reduce by half the requirement for animals in such research.

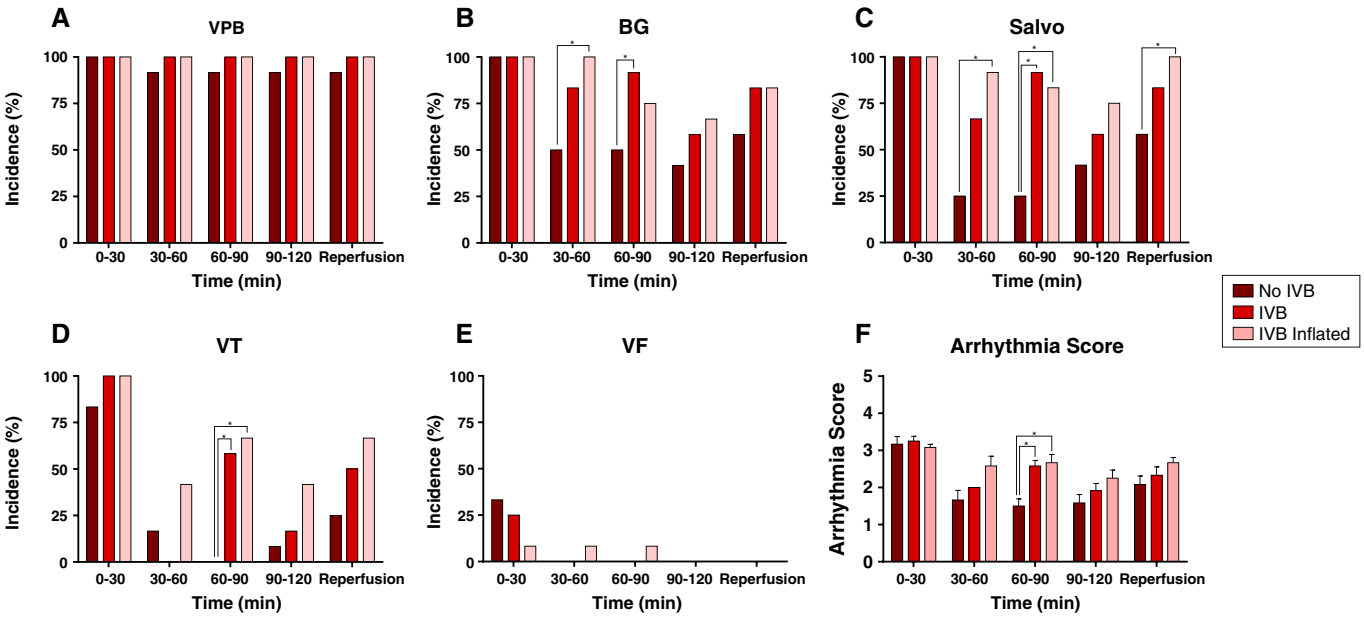

Figure 3

Occurrence (% incidence per group) of increasingly severe ventricular arrhythmias (VPB to VF) and five-point summary arrhythmia score (mean ± SEM) during 120 min ischaemia in hearts with *small* IZs. There were $n = 12$ hearts in each group. *$P < 0.05$.

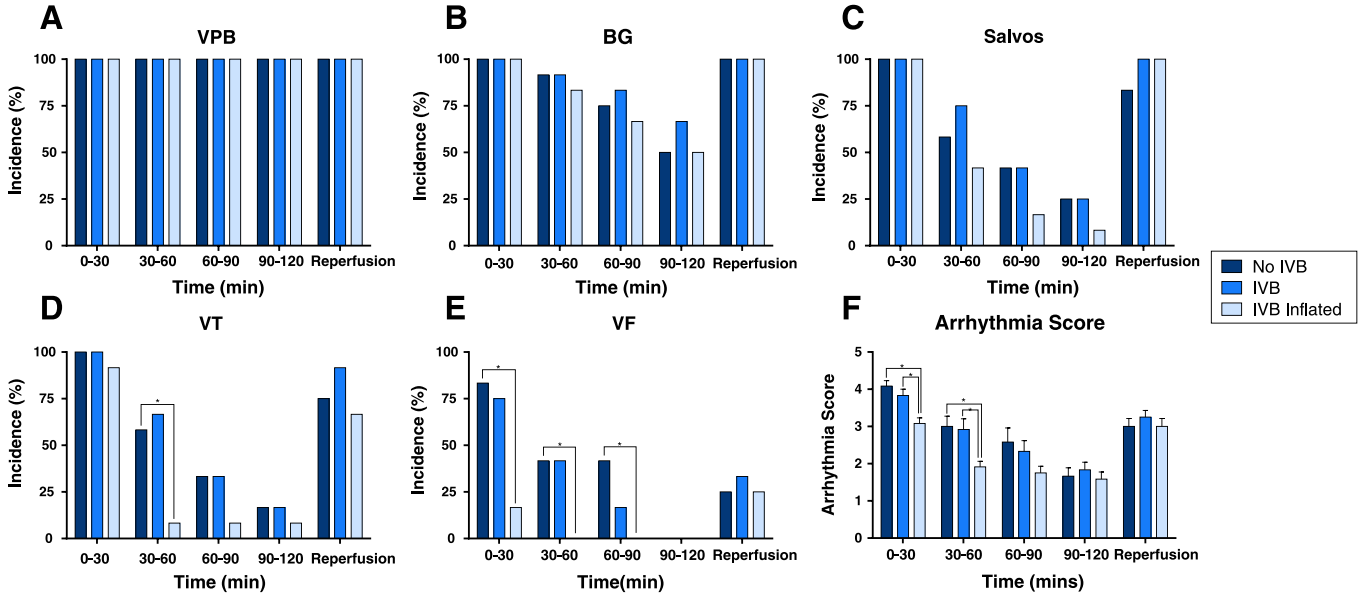

Figure 4

Occurrence (% incidence per group) of increasingly severe ventricular arrhythmias (VPB to VF) and five-point summary arrhythmia score (mean ± SEM) during 120 min ischaemia in hearts with *large* IZs. There were $n = 12$ hearts in each group. *$P < 0.05$.

Previous studies suggest that increased ventricular load is arrhythmogenic (Franz *et al.*, 1989; Hansen *et al.*, 1990; Franz *et al.*, 1992; Chorro *et al.*, 2000; Coronel *et al.*, 2002; Parker *et al.*, 2004), albeit few attempts have been made to test directly the effect of function assessment on *ischaemia*-induced VF (most studies used other endpoints such as VT or electrically induced arrhythmias). In the most relevant study, in Langendorff perfused pig hearts, Coronel *et al.* (2002) found that IVB inflation increased ischaemia-induced VT versus no IVB, an effect attributed to an arrhythmogenic effect of 'wall stress' (Coronel *et al.*, 2002). However, VF incidence was not facilitated. Moreover, baseline arrhythmia susceptibility in IVB-free hearts was exceptionally low. The influence of the size of IZ, a factor known to determine VF incidence in isolated hearts (Ridley *et al.*, 1992), was not considered. Therefore, it is not possible to make direct comparison with the

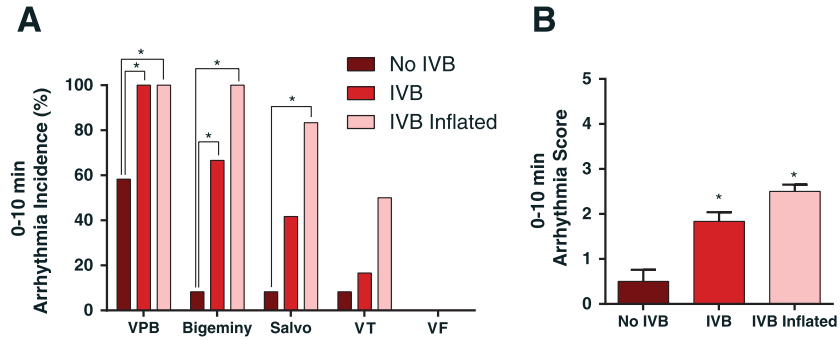

Figure 5

Occurrence (% incidence per group) of increasingly severe ventricular arrhythmias (VPB to VF) (part A) and five-point summary arrhythmia score (mean ± SEM) (part B) during the first 10 min of ischaemia in hearts with *small* IZs. There were $n = 12$ hearts in each group. *$P < 0.05$ (A); *$P < 0.05$ versus No IVB (B).

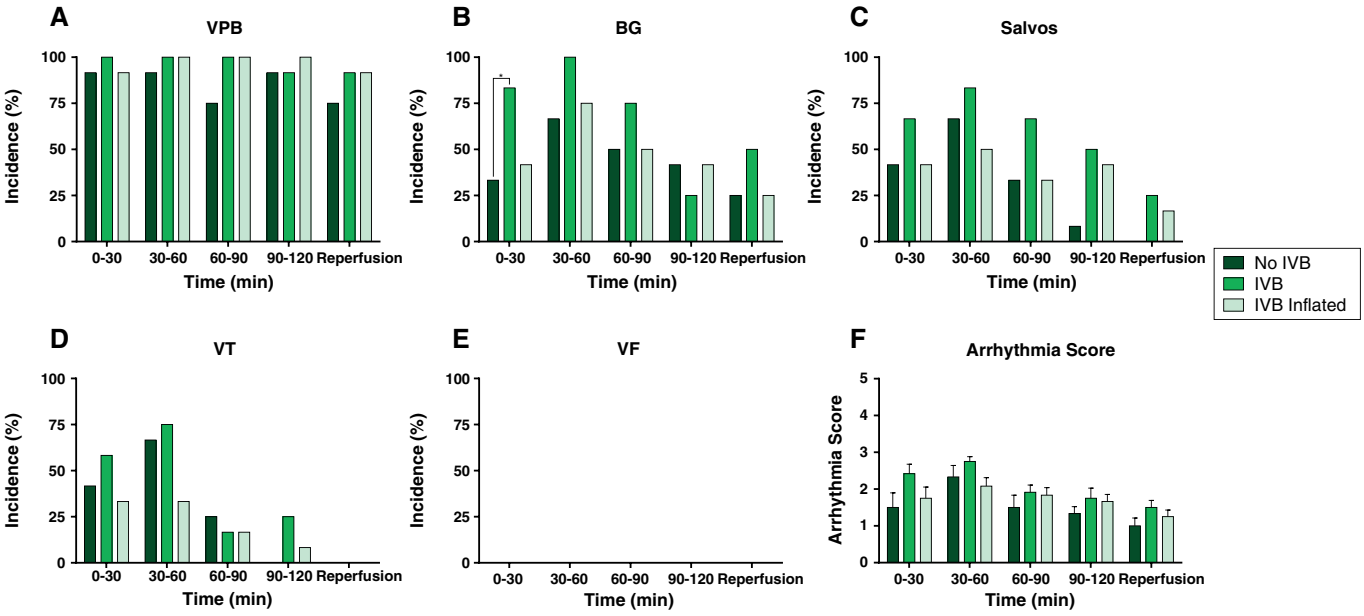

Figure 6

Occurrence (% incidence per group) of increasingly severe ventricular arrhythmias (VPB to VF) and five-point summary arrhythmia score (mean ± SEM) during 120 min ischaemia in hearts with *large* IZs + verapamil. There were $n = 12$ hearts in each group. *$P < 0.05$.

present study in which we addressed these issues by testing the effects of IVB inflation with different predetermined IZ sizes, including large IZs sufficient to evoke a high susceptibility to VF.

We found that when control VF incidence was high (hearts with large IZs), inflation of an IVB *reduced* the incidence of ischaemia-induced VF substantially, from 83 to 17%. This is a novel finding. However, interestingly, we found that less severe arrhythmias such as VPBs occurred sooner and lasted longer with IVB inflation, which accords with the study of Coronel *et al.* in isolated pig hearts (Coronel *et al.*, 2002). This appears to suggest that IVB inflation has two effects, hastening and facilitating the less severe ischaemia-induced arrhythmias, yet suppressing the occurrence of VF. This does not contradict Coronel *et al.* (2002), who had had no scope to consider VF suppression owing to their low arrhythmia susceptibility in non-IVB hearts. The present data do not, however, accord with findings of Hatcher and Clements-Jewery (2012), who report a high incidence of ischaemia-induced VF in perfused rat hearts containing an IVB. It is not possible to speculate on an explanation, however, because the latter finding has, to date, been reported only in brief in a meeting abstract, and in any case, the study was not designed to systematically examine the effects of an IVB and its inflation on VF.

Neither the IVB nor its inflation impaired the ability of an established antiarrhythmic concentration of verapamil (Farkas *et al.*, 1999) to abolish VF. This means that the effect

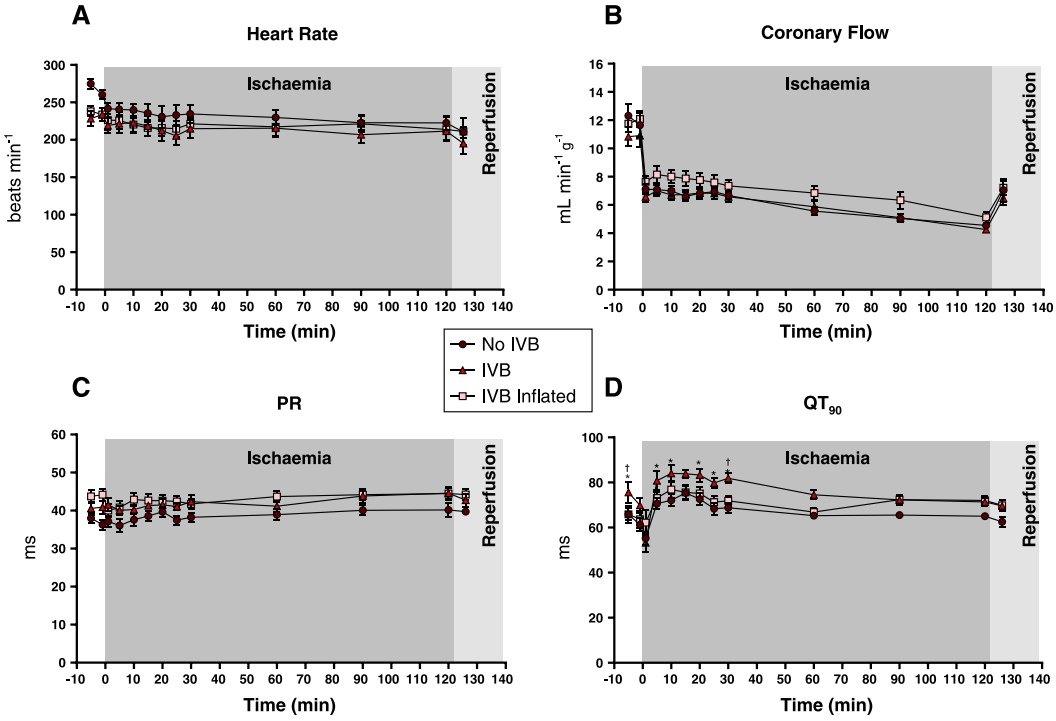

Figure 7

Heart rate (part A), coronary flow (part B), PR (part C) and QT_{90} intervals (part D) (mean ± SEM) in hearts with *small* IZs. There were $n = 12$ hearts in each group. *$P < 0.05$ versus No IVB; †$P < 0.05$ versus IVB inflated.

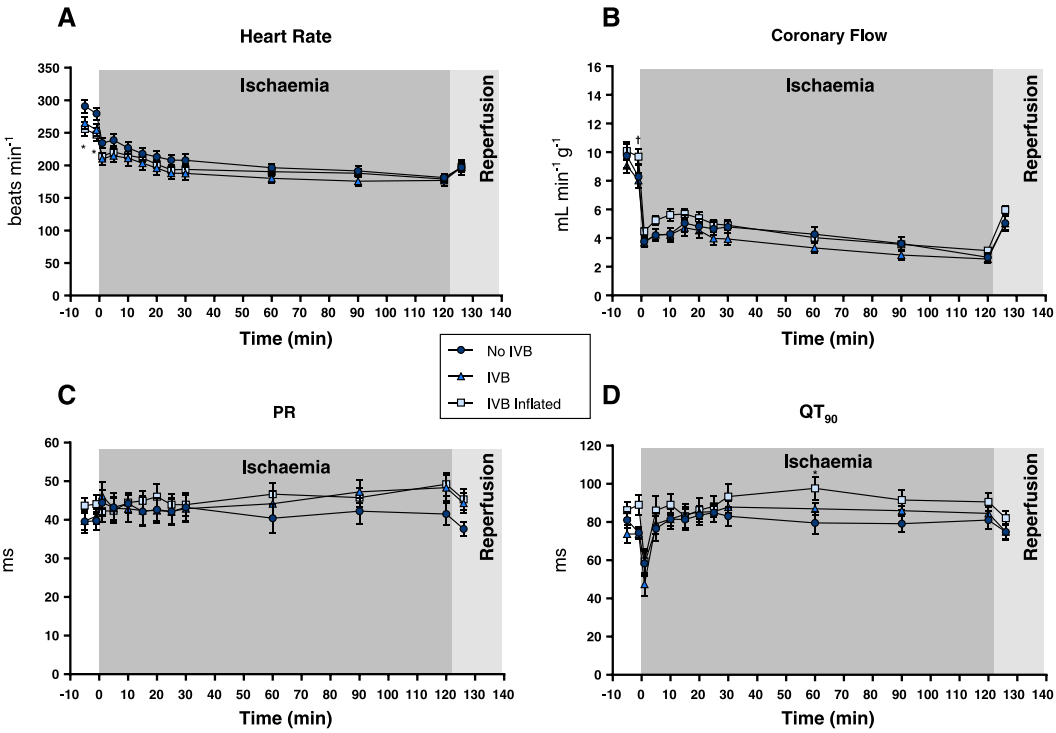

Figure 8

Heart rate (part A), coronary flow (part B), PR (part C) and QT_{90} intervals (part D) (mean ± SEM) in hearts with *large* IZs. There were $n = 12$ hearts in each group. *$P < 0.05$ versus No IVB ; †$P < 0.05$ versus IVB inflated.

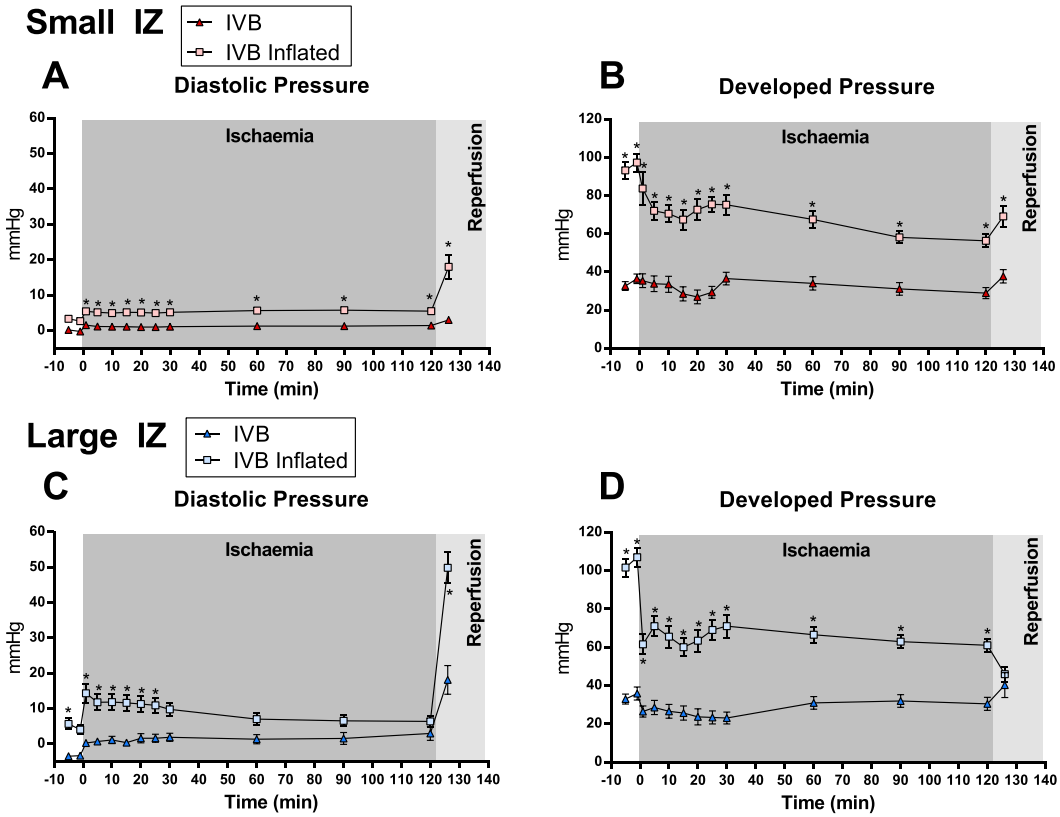

Figure 9

Diastolic and developed LV pressures (mean ± SEM) from hearts with small (A–B) and large (C–D) IZs. There were $n = 12$ hearts in each group. *$P < 0.05$ versus IVB.

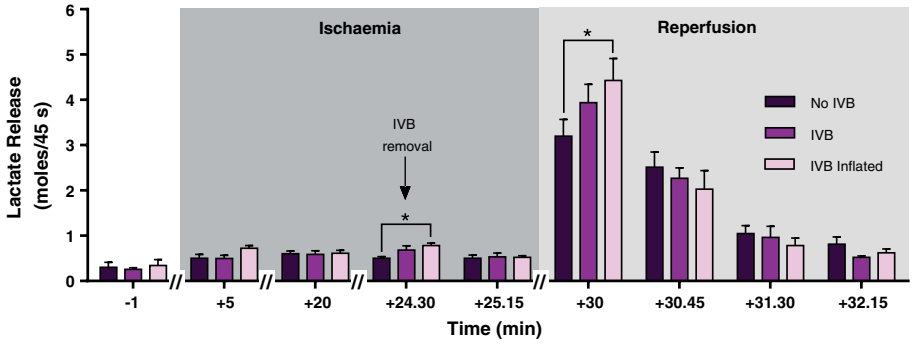

Figure 10

Changes in lactate release (mean ± SEM) in sequential 15 s coronary effluent samples taken at the intervals shown before (−1), during and after 30 min of ischaemia. There were $n = 5$ hearts per group. Only seven hearts ($n = 2$ or 3 per group) had measurements taken for +5 min owing to a temporary design oversight. *$P < 0.05$.

of IVB inflation is potentially disadvantageous in ischaemia-induced arrhythmia studies more in terms of its effects on the power of the bioassay to detect effects of antiarrhythmic drugs (reduced because VF incidence is low) rather than by changing the response to an effective drug. This contrasts with findings on the effects of IVB inflation on the actions of the same drug on *electrically* induced arrhythmias (Chorro *et al.*, 2000). This emphasizes the importance of selecting appropriate endpoints and not relying on an unvalidated surrogate for ischaemia-induced VF, such as electrically induced VT. We note that, in humans, electrically induced arrhythmias do not predict subsequent effectiveness of drugs in patients at risk of ischaemia-induced VF (Bourke *et al.*, 1995; Goldstein *et al.*, 1995).

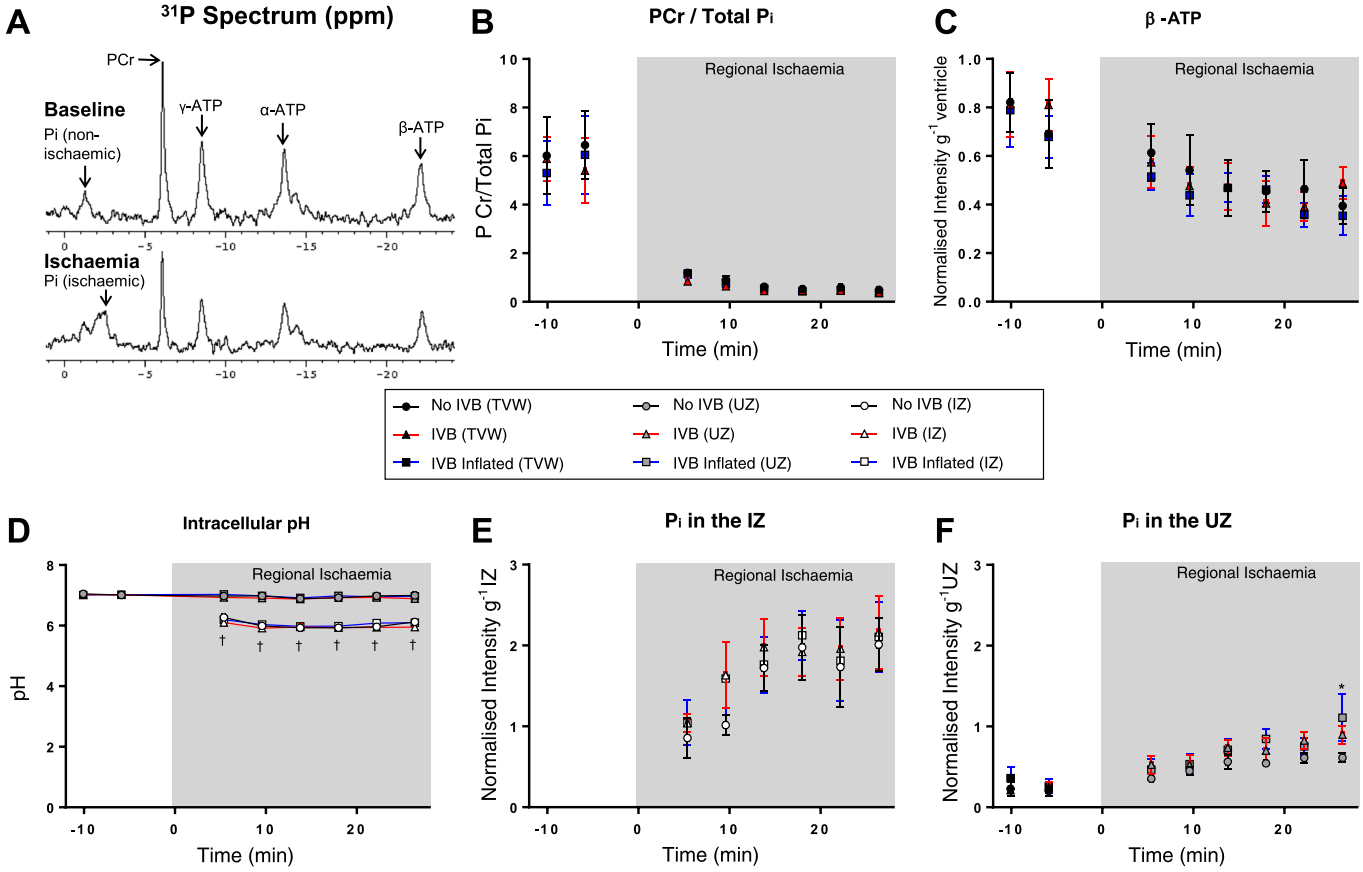

Figure 11

An example of ^{31}P NMR spectra from an IVB inflated heart at baseline before ischaemia (A, top part) and after 25 min ischaemia (A, bottom part) showing the development of the split P_i peak. Group data (mean ± SEM), all $n = 5$ per group, show PCr to total P_i ratio (part B), β-ATP (part C), intracellular pH (part D) P_i in the IZ (part E) and P_i in the UZ (part F). †$P < 0.05$ for each IZ value versus each time-matched UZ value; *$P < 0.05$ versus No IVB.

Possible mechanisms of IVB inflation-induced VF suppression

Several mechanisms may be ruled out. It is important to emphasize the VF was ameliorated only by IVB *inflation* and not by the physical presence of the (minimally inflated) IVB. LV stretch may hasten repolarization and shorten QT interval (Eckardt *et al.*, 2001; Lerman *et al.*, 2001). In the present study, the IVB, whether inflated or not, had no appreciable or consistent effect on ancillary readouts including QT_{90} interval in hearts with large IZs when VF suppression was significant. It is therefore unfeasible to attribute IVB-induced VF *suppression* to a stretch-induced hastening of repolarisation.

IVB inflation has the potential to evoke stretch-induced nitric oxide (NO) release (Liao *et al.*, 2006; Hooper *et al.*, 2013; Pullen *et al.*, 2014). However, previous data have shown NO and its precursor, L-arginine, have little or no effect on ischaemia-induced VF (Pabla and Curtis, 1995; Wainwright *et al.*, 2002), so this explanation is unfeasible.

A more plausible explanation relates to IVB-induced compression of coronary arteries leading to endo/subendocardial ischaemia. This is possible only in the IVB *inflated* group, primarily during systole, when systolic LV pressure (>100 mmHg before ischaemia) exceeds perfusion pressure (80 mmHg). Coronel *et al.* (2002) contended that their inflating an IVB to generate a systolic pressure of only 30–50 mmHg meant that perfusion pressure (>50 mmHg) was sufficient to 'prevent subendocardial' ischaemia. In the present study, IVB inflation was set higher to generate physiologically relevant LV developed pressures, but diastolic pressure was always tens of mmHg below perfusion pressure, meaning coronary flow would be unimpeded during diastole. Indeed, global coronary flow (averaged over a minute) was not reduced by IVB inflation (Figure 8). Despite this, it is possible that IVB inflation induced *localized* endo/subendocardial ischaemia during a proportion of the cardiac cycle as a consequence of localized tissue compression in the vicinity of the balloon, especially during systole. Although endo/subendocardial vascular compression would reduce flow locally, the positive inotropy resulting from balloon inflation would indirectly increase flow in midmyocardium or epicardium due to inotropy-related hyperaemia (Elhendy *et al.*, 2002) accounting for the maintenance of global flow. In view of this, we sought direct evidence of IVB inflation-induced localized ischaemia.

Coronary effluent lactate levels are increased by ischaemia (Stanley *et al.*, 1997). In the present study, there was a small baseline release of lactate during normal perfusion before regional ischaemia, indicative of normal aerobic metabolism

in a working muscle. There was very little increase in effluent lactate during regional ischaemia. This is consistent with the trapping in the ischaemic region of any ischaemia-induced increase in lactate production, a consequence of the very low level of collateral vessels in the rat heart, which are capable of delivering no more than 5% of normal coronary flow during regional ischaemia (Maxwell *et al.*, 1987). We found a transient increase in effluent lactate following IVB removal during maintained regional ischaemia, but only if the IVB had been previously inflated (i.e. in the group with VF suppression). Release of the coronary ligature to cause reperfusion of the ischaemic region also induced lactate release, albeit of many times greater magnitude than at baseline or during IVB deflation and removal. Clearly these transient increases in coronary lactate during IVB deflation, and removal and during release of the coronary ligature reflect *reperfusion of ischaemic myocardium*, with a very small region of myocardium reperfused by IVB deflation, and a very large region reperfused after release of the coronary ligature. This implies that IVB inflation caused a localized thin layer of ischaemia encompassing the endo/subendocardium of the UZ – the nominally non-ischaemic region of the heart – by compression of endocardial and subendocardial vessels of the UZ. Then, with IVB deflation, these vessels become fully reperfused, and any accumulated lactate passes into the coronary effluent. The IVB itself is not implicated because its presence, uninflated, did not suppress VF and its removal did not release lactate.

The reason endo/subendocardial ischaemia would reduce regional ischaemia-induced VF incidence is because the area of interface at the border of the ischaemic and uninvolved regions would be reduced. This is the site of arrhythmogenesis (Ridley *et al.*, 1992). Nevertheless, the large effect of IVB inflation on VF incidence is quite remarkable given the evidence, described above, that the extent of IVB-induced ischaemia is small.

One argument against this mechanism of VF suppression is that if IVB inflation reduced flow, it would do so only during systole, as noted above. Consequently, there would be coronary flow throughout the heart during diastole, as occurs *in vivo*. Nevertheless, if diastolic perfusion were adequate to prevent IVB compression-induced ischaemia, it would become difficult to account for the washout of lactate after IVB deflation. Simple mechanical damage invoked by IVB does not explain this because it would not be *reversible* by IVB removal.

Our interpretation of the lactate data was confirmed by the ^{31}P NMR spectroscopy data. IVB inflation caused an increase in the size of the P_i value derived from the UZ peak, which reached statistical significance 25 min after the start of regional ischaemia. The value of this UZ peak (and the corresponding UZ peaks for the other two groups) were all lower than corresponding values recorded from the IZ (compare Figure 11E and F). Indeed, in the 'no IVB' group, the UZ P_i values during ischaemia at no time increased above pre-ischaemia values (Figure 11F). Before ischaemia, PCr, β-ATP, intracellular pH and P_i values were all normal, and during ischaemia, values changed in accordance with published findings (Bottomley *et al.*, 1987; Ishibashi *et al.*, 1993; Cross *et al.*, 1995; Takaoka *et al.*, 1999) with no significant differences between groups. These findings are entirely consistent with generation of *partial* ischaemia in the UZ (localized and small in extent, presumably endocardial because total UZ coronary flow was not lowered versus the no IVB and IVB groups) as a consequence of IVB inflation. Although an increase in P_i is normally interpreted to indicate ischaemia, it is more accurately reflective of a net imbalance between ATP synthesis and ATP utilization, which can occur even if the 'ischaemia' present is insufficient to affect intracellular pH, as in the present study. The ischaemia is partial because the P_i peak that increased in size is separate from the acid-shifted peak from the same hearts that represents the IZ peak (Figure 11A). This finding accords with the lactate data and confirms that although IVB inflation has no adverse consequences in the normally perfused heart, when regional ischaemia elevates end diastolic pressure, IVB inflation causes localized vascular compression in the UZ. Each heartbeat in the IVB inflated group would therefore be associated with impaired endocardial coronary flow during diastole. This accounts for the significant but almost imperceptible changes in cardiac biochemistry that we observed. These changes appear to be sufficient to greatly reduce the arrhythmogenicity of regional ischaemia.

This is the first report, to our knowledge, of *regional* intracellular P_i changes during regional ischaemia measured by ^{31}P NMR spectroscopy in isolated perfused hearts. It is noteworthy that the extent, and the progression of the depth of ischaemia over time in the IZ itself, in these collateral deficient hearts (Curtis, 1998), appears to be entirely determined by the *regional* loss of blood flow (in the IZ), with no significant hastening of ischaemia in the IZ territory by IVB inflation. Given that IVB inflation causes hearts to generate greater force (inotropy), this would imply that it is not possible to alleviate the severity of regional ischaemia in the IZ in collateral deficient hearts by use of negative inotropes, and hence delay the development of infarction by such an approach. Likewise, IVB inflation, and hence positive inotropy, did not change P_i or ATP before ischaemia, during normal perfusion, suggesting that isolated hearts perfused with Krebs solution are not on the brink of ischaemia due to the poor oxygen delivery capacity of water (Krebs solution) versus blood.

Thus, reversible coronary compression sufficient to cause localized block of coronary flow during systole and impair but not prevent it during diastole, leading to a layer of underperfused tissue abutting the coronary ligation-induced region of ischaemia, reducing its arrhythmogenicity (by impairing endocardial conduction), remains the only plausible explanation for the measured changes in lactate, and the suppression of VF.

There is important independent support for the hypothesis that impairing endocardial conduction has antiarrhythmic activity. Arrhythmia suppression by deliberate endocardial injury has been demonstrated in dogs (Chilson *et al.*, 1986; Damiano *et al.*, 1986; Janse *et al.*, 1986). Chemical ablation with phenol of a thin layer of ventricular endocardium in dog hearts *in vitro* suppresses ischaemia-induced VF (Janse *et al.*, 1986). Additionally, in humans, endocardial resection in patients with an aneurysm can suppress ventricular arrhythmias after hospital discharge (Horowitz *et al.*, 1980).

Key implications

The important finding from the present study is the robust evidence for IVB inflation-induced suppression of VF, which

has extremely important research implications. Disappointingly, the data imply that it would be unwise to incorporate IVB pressure recordings into future arrhythmia research protocols. The data do nevertheless confirm a largely neglected, likely importance of the endocardium in arrhythmogenesis, specifically in the initiation of ischaemia-induced VT and VF, and the antiarrhythmic effect of ablation of the endocardium.

Future work

Techniques such as cardiac imaging and use of microspheres may shed more light on the location and extent of regional underperfusion associated with IVB inflation. However, it is debatable whether further research is necessary or justified. The original intent of the study was to determine whether animal usage could be reduced by a dual approach of simultaneous rhythm and function assessment during regional ischaemia in the medium-throughput Langendorff preparation. Regardless of the mechanism of arrhythmia suppression by IVB inflation, our findings preclude any further attempt to elaborate this approach, and the mechanism of VF suppression is largely immaterial.

Acknowledgements

We thank Professor David J Hearse and the Burton Fund for supporting this study.

Author contributions

C. D. E. W., B. A. O., T. R. E., R. M. and D. Y. performed the research. M. J. C., C. D. E. W., B. A. O. and T. R. E. designed the research study. C. D. E. W., B. A. O. and T. R. E. analysed the data. C. D. E. W., B. A. O., T. R. E. and M. J. C. wrote the paper.

Conflict of interest

Authors declare that they have no conflict of interest.

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