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ORIGINAL ARTICLE

Recirculating cardiac delivery of AAV2/1SERCA2a improves myocardial function in an experimental model of heart failure in large animals

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Abstract

Abnormal excitation–contraction coupling is a key pathophysiologic component of heart failure (HF), and at a molecular level reduced expression of the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a) is a major contributor. Previous studies in small animals have suggested that restoration of SERCA function is beneficial in HF. Despite this promise, the means by which this information might be translated into potential clinical application remains uncertain. Using a recently established cardiac-directed recirculating method of gene delivery, we administered adeno-associated virus 2 (AAV2)/1SERCA2a to sheep with pacing-induced HF. We explored the effects of differing doses of AAV2/1SERCA2a (low 1×10^{10} d.r.p.; medium 1×10^{12} d.r.p. and high 1×10^{13} d.r.p.) in conjunction with an intra-coronary delivery group (2.5×10^{13} d.r.p.). At the end of the study, haemodynamic, echocardiographic, histopathologic and molecular biologic assessments were performed. Cardiac recirculation delivery of AAV2/1SERCA2a elicited a dose-dependent improvement in cardiac performance determined by left ventricular pressure analysis, ($+dP/dt_{max}$; low dose -220 ± 70, $P>0.05$; medium dose 125 ± 53, $P<0.05$; high dose 287 ± 104, $P<0.05$) and echocardiographically (fractional shortening: low dose -3 ± 2, $P>0.05$; medium dose 1 ± 2, $P>0.05$; high dose 6.5 ± 3.9, $P<0.05$). In addition to favourable haemodynamic effects, brain natriuretic peptide expression was reduced consistent with reversal of the HF molecular phenotype. In contrast, direct intra-coronary infusion did not elicit any effect on ventricular function. As such, AAV2/1SERCA2a elicits favourable functional and molecular actions when delivered in a mechanically targeted manner in an experimental model of HF. These observations lay a platform for potential clinical translation.
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Keywords: *heart failure; gene delivery; adeno-associated virus; calcium handling; contractility; dose-response*

Introduction

Despite pharmacologic advances in the treatment of heart failure (HF), mortality and morbidity remain unacceptably high. Moreover, for patients with end-stage HF therapeutic options are limited to heart transplantation and possibly long-term mechanical circulatory support. However, these options are not appropriate for many patients with advanced HF when associated with other co-morbid diseases. In this context, alternative approaches such as gene and cell therapy have attracted increased attention. Although transfer of pathophysiologically relevant genes has shown to be promising,[1–4] significant ongoing issues related to delivery, including vector efficiency, dose, specificity/tropism and safety are areas of concern. The use of adeno-associated virus (AAV) vectors to deliver genes to cardiomyocytes has been shown to be highly efficient both *in vitro* and *in vivo*.[1–3] However, although there is continued development of AAV serotypes for improved cell/tissue-type targeting,[5] the issue remains of systemic delivery and safety.[6] As a consequence, delivery methods that result in homogeneous expression of the gene that are safe and well targeted need to be developed.

The reduction in contractile function evident in HF has been well documented with defects in calcium signalling seen as one of the key pathophysiological aspects of the failing heart. In particular there is decreased expression of sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a) which contributes to the reduced SR Ca^{2+} accumulation and abnormal calcium cycling.[7] As such, increasing SERCA2a expression by gene transfer in isolated cardiomyocytes and rodent models of HF has been shown to improve calcium handling and contractility in cardiomyocytes.[4,8,9] However, limitations in effective cardiac gene transfer methods have limited the ability to test the over expression in large animals.

To combat the need for more targeted, homogeneous delivery of agents we have developed a percutaneous delivery system that enables isolation of the cardiac circulation for targeted delivery of therapeutic agents, in this case AAV vectors, to the heart. Recently, we developed an effective, well-tolerated method for achieving

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homogeneous perfusate distribution in the failing large animal heart.[10] In this study we showed that the recirculating cardiac delivery of an adenovirus encoding a pseudo-phosphorylated mutant of phospholamban improved left ventricular function in animals with pacing-induced HF.[10] Moreover, by isolating delivery to the heart systemic exposure was limited.

In this study we addressed several key issues relating to the potential for gene therapy-based treatment of HF. Specifically, we evaluated the effectiveness and dose dependence of *SERCA2a* gene delivery using AAV2/1SERCA2a in experimental HF. In conjunction, we compared the effectiveness of the recirculating delivery approach and direct intra-coronary infusion.

Results

As shown in Table 1, haemodynamic and echocardiographic data indicate that the study groups were generally well matched before AAV2/1 administration. By chance, the low-dose group did demonstrate mildly, but significantly greater ventricular function ($P<0.05$) compared to the recirculation high-dose and medium-dose groups as well as the control animals.

A total of 11 animals were excluded from the final analysis as a result of experimental outcomes and variables. Five animals died during the study, one from each group with the exception of the recirculation high-dose and intra-coronary delivery groups. As a result of anatomical differences, one animal was excluded due to difficulty in delivering the vector. Four animals had pacemaker malfunction or infection requiring early removal from the study and two animals were excluded due to confounding haemodynamic parameters, one animal with excessive hypertension and one with severe mitral regurgitation confounding haemodynamic assessment. No ventricular arrhythmias were noted during haemodynamic evaluation, however long-term telemetric recording was not performed.

Efficacy

Echocardiographic parameters. There was no significant difference between control and the low-dose recirculation group for all echocardiographic parameters (Figure 1a). The medium dose of AAV2/1SERCA2a prevented left ventricular remodelling as reflected by a significant reduction in left ventricular internal diameter (Figure 2a, $P<0.05$) compared to control group. In conjunction there was a trend for improvement in both fractional shortening (FS) and ejection fraction (EF; Figures 1c and d).

In the 6-week follow-up animals, the left ventricular (LV) internal diameters in both systole and diastole did not differ between the three groups: control, recirculation and intra-coronary delivery (Figures 2a and b). Both FS and EF were significantly improved in the high-dose recirculation group compared to intra-coronary delivery despite a 2.5-fold lower dose (Figures 2c and d, $P=0.05$).

Analysis of the data incorporating animals with confounding haemodynamic parameters ($n=2$, severe MR and excessive hypertension) resulted in no significant difference in the change from baseline for FS between control (0 ± 0.9), low dose (-3 ± 1.7) and medium dose (1 ± 1.8) after 4 weeks pacing ($P>0.05$).

Left ventricular pressure parameters. There was no significant difference between control and the low-dose recirculation group for all haemodynamic parameters (Figure 3). A significant improvement in both $+dP/dt_{max}$ (Figure 3a, $P<0.05$) and $-dP/dt_{max}$ (Figure 3b, $P<0.05$) was observed in the medium-dose recirculation group of animals compared to both controls and the low-dose recirculation animals. There was no significant difference between the groups for end diastolic pressure (EDP, control -2 ± 1.7 mm Hg; low dose -3 ± 2.2 mm Hg; medium dose -1 ± 1.4 mm Hg) or mean arterial pressure (MAP, control 9 ± 4.2 mm Hg; low dose -1 ± 4.2 mm Hg; medium dose 4 ± 4.6 mm Hg). End systolic pressure (ESP) was significantly greater in the medium group

Table 1 Functional characteristics of the groups following randomization before delivery of the vector

	4 weeks			*6 weeks*		
	Control	*V-Focus low dose*	*V-Focus medium dose*	*Control*	*V-Focus high dose*	*Intra-coronary*
Echo						
FS (%)	19 ± 1.0	22 ± 1.1[¥]	18 ± 1.3	20 ± 3.3	13 ± 1.8	17 ± 1.9
LV Ids (mm)	42 ± 0.6	42 ± 1.1	45 ± 1.3	42 ± 2.8	43 ± 1.9	40 ± 2.6
LV Idd (mm)	52 ± 0.7	55 ± 1.7	56 ± 1.7	53 ± 2.3	50 ± 2.9	49 ± 2.5
EF (%)	35 ± 1.6	40 ± 1.8*,#	33 ± 2.1	37 ± 5.4	25 ± 3.1	32 ± 3.2
Hemodynamics						
$+dP/dt_{max}$	744 ± 44.1	958 ± 62.5*,#	865 ± 55.2	814 ± 121.5	615 ± 98.6	771 ± 75.9
$-dP/dt_{max}$	1352 ± 68.2	1607 ± 58.7*	1371 ± 76.5	1396 ± 127.2	1268 ± 167.3	1410 ± 102.9
EDP (mm Hg)	17 ± 1.7	15 ± 1.9	16 ± 2.4	18 ± 5.3	17 ± 2.7	12 ± 2.2
ESP (mm Hg)	101 ± 3.9	106 ± 2.5	107 ± 4.9	104 ± 4.8	91 ± 6.3	100 ± 4.5
MAP (mm Hg)	94 ± 4.1	92 ± 9.2	94 ± 9.6	100 ± 2.6	95 ± 4.4	96 ± 4.9
n	13	11	11	5	7	6

Abbreviations: EDP, end diastolic pressure; EF, ejection fraction; ESP, end systolic pressure; FS, fractional shortening; LV Idd, left ventricular internal diameter diastole; LV Ids, left ventricular internal diameter systole; MAP, mean arterial pressure.
Data expressed as mean ± s.e.m.
*$P<0.05$ compared to control.
#$P<0.05$ compared to V-Focus medium dose (1×10^{12} d.r.p.).
¥$P<0.05$ compared to V-Focus high dose (1×10^{13} d.r.p.).

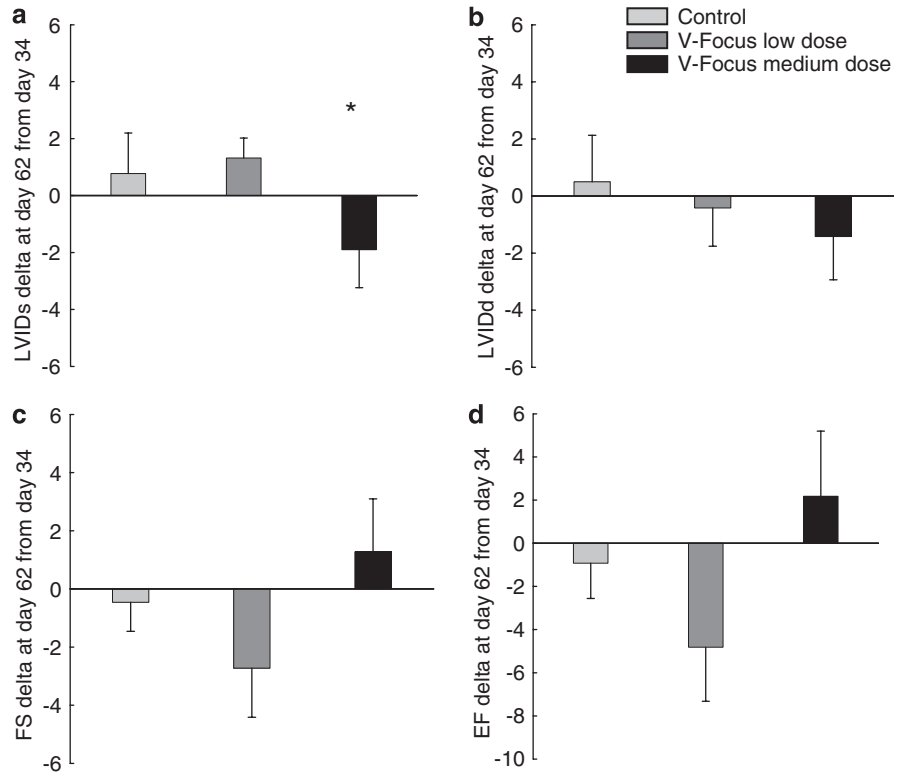

Figure 1 Echocardiographic parameters for animals treated with low-dose (1×10^{10} d.r.p.) or medium-dose (1×10^{12} d.r.p.) adeno-associated virus 2 (AAV2)/1SERCA2a (sarcoplasmic reticulum Ca^{2+} ATPase) shown as a change from pre-delivery and 4 weeks post-delivery with the V-Focus device. (**a**) Left ventricular (LV) internal diameter in systole, (**b**) LV internal diameter in diastole, (**c**) LV fractional shortening (FS) and (**d**) LV ejection fraction (EF). Values are mean ± s.e.m., $^*P < 0.05$ compared to control group.

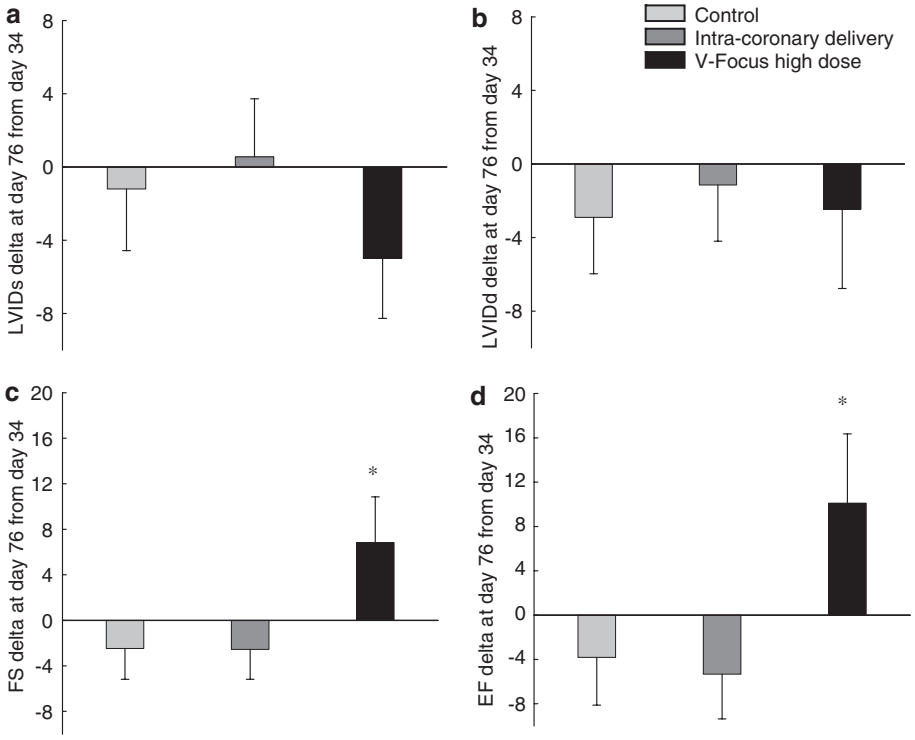

Figure 2 Echocardiographic parameters for animals treated with V-Focus high dose (1×10^{13} d.r.p.) or intra-coronary delivery (2.5×10^{13} d.r.p.) adeno-associated virus 2 (AAV2)/1SERCA2a (sarcoplasmic reticulum Ca^{2+} ATPase) shown as a change from pre-delivery and 6 weeks post-delivery. (**a**) Left ventricular (LV) internal diameter in systole, (**b**) LV internal diameter in diastole, (**c**) LV fractional shortening (FS) and (**d**) LV ejection fraction (EF). Values are mean ± s.e.m., $^*P = 0.05$ compared to intra-coronary delivery.

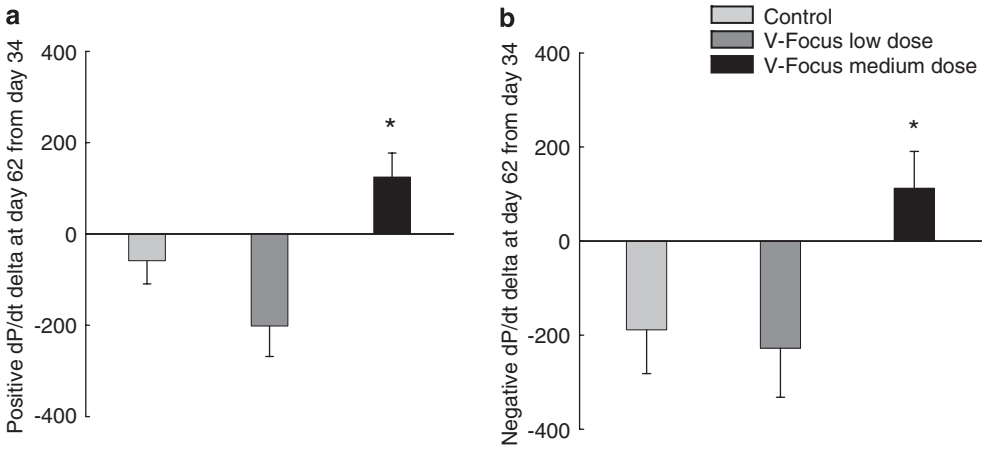

Figure 3 Left ventricular (LV) pressure derivatives parameters for animals treated with low-dose (1×10^{10} d.r.p.) or medium-dose (1×10^{12} d.r.p.) adeno-associated virus 2 (AAV2)/1SERCA2a (sarcoplasmic reticulum Ca^{2+} ATPase) shown as a change from pre-delivery and 4 weeks post-delivery with the V-Focus device. (**a**) LV $+dP/dt_{max}$, (**b**) LV $-dP/dt_{max}$. Values are mean ± s.e.m., $^*P < 0.05$ compared to control group and V-Focus low dose.

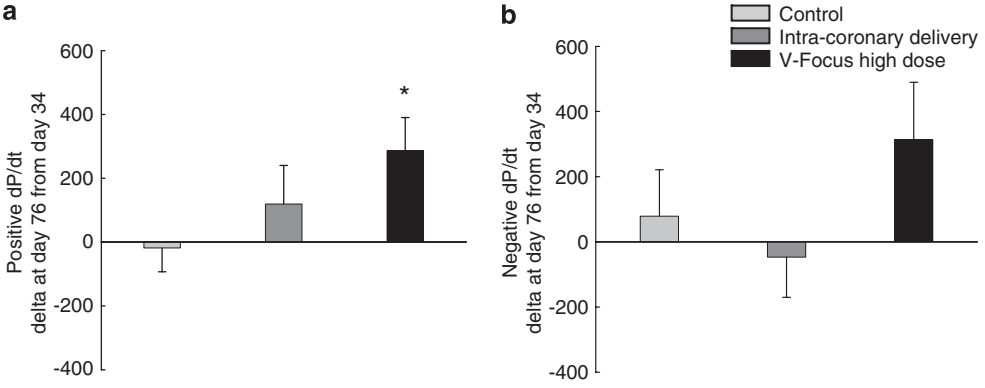

Figure 4 Left ventricular (LV) pressure derivatives parameters for animals treated with V-Focus high dose (1×10^{13} d.r.p.) or intra-coronary delivery (2.5×10^{13} d.r.p.) adeno-associated virus 2 (AAV2)/1SERCA2a (sarcoplasmic reticulum Ca^{2+} ATPase) shown as a change from pre-delivery and 6 weeks post-delivery. (**a**) LV $+dP/dt_{max}$, (**b**) LV $-dP/dt_{max}$. Values are mean ± s.e.m., $^*P < 0.05$ compared to control.

compared to control animals (control −8 ± 3.6 mm Hg; low dose −4 ± 6.6 mm Hg; medium dose 10 ± 5.7 mm Hg, $P < 0.05$).

In the 6-week group of animals a significant improvement in $+dP/dt_{max}$ was seen in the high-dose recirculation group compared to control (Figure 4a, $P < 0.05$) with no difference in $-dP/dt_{max}$ between these groups ($P > 0.05$). Intra-coronary delivery of AAV2/1SERCA2a did not result in a significant improvement in either $+dP/dt_{max}$ or $-dP/dt_{max}$ (Figure 4b, $P > 0.05$) compared to control animals. There was no significant difference in change from randomization for EDP (control −3 ± 7.4 mm Hg; high dose −2 ± 3.4 mm Hg; intra-coronary 0 ± 2.2 mm Hg), ESP (control 3 ± 6.4 mm Hg; high dose 13 ± 3.1 mm Hg; intra-coronary 5.8 ± 4.4 mm Hg) or MAP (control −1 ± 4.5 mm Hg; high dose 8 ± 3.0 mm Hg; intra-coronary 8 ± 3.2 mm Hg) between the groups.

Tissue BNP and human SERCA2a expression. Myocardial brain natriuretic peptide (BNP) expression 6 weeks after AAV2/1SERCA2a relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was reduced by 6.7-fold (ΔC_t intra-coronary vs V-Focus: 5.7 ± 0.6 vs 8.4 ± 0.9, $P < 0.05$) in animals receiving gene therapy via recirculating compared to intra-coronary delivery. SERCA2a mRNA expression was also determined 6 weeks after administration of the AAV2/1SERCA2a. In comparison to the animals receiving intra-coronary AAV2/1SERCA the V-Focus group of animals exhibited significantly a 29-fold greater human SERCA (hSERCA) mRNA expression (ΔC_t intra-coronary vs V-Focus: 11.4 ± 0.9 vs 6.5 ± 1.2, $P < 0.05$).

Safety

Histopathology. As shown in Table 2, there was no significant difference in levels of fibrosis between animals that received AAV2/1SERCA2a or the control groups. Any fibrosis that did occur was considered minimal (graded 1 or 2 on the scale of 1–5) and did not exhibit any trends or patterns in distribution either within individual hearts or between groups. With respect to chronic inflammation of the myocardium, there was no significant difference in either the incidence or the severity of inflammation observed between groups at either 4 or 6 weeks follow-up (Table 2).

Table 2 Histopathalogical and clinical pathology characteristics of animals

	4-week follow-up			*6-week follow-up*		
	Control	*V-focus low dose*	*V-focus medium dose*	*Control*	*V-focus high dose*	*Intra-coronary*
Histopathology						
Fibroplasia						
Number observed	0/10	2/7	0/8	0/6	0/7	0/7
Degree (1–5)	—	1	—	—	—	—
Inflammation						
Number observed	10/10	6/7	7/8	6/6	7/7	7/7
Degree (1–5)	1.1 ± 0.1	1.3 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Clinical pathology						
Haematology						
RBC	8.5 ± 0.4	8.5 ± 0.2	8.5 ± 0.4	9.2 ± 0.3	8.5 ± 0.2	8.6 ± 0.3
WBC	4.5 ± 0.3	5.6 ± 0.4	5.6 ± 0.7	6.6 ± 0.5	5.3 ± 0.5	5.1 ± 0.3*
Lymphocytes	1.9 ± 0.3	3.5 ± 0.4*	2.1 ± 0.4#	4.1 ± 0.5	2.6 ± 0.3*	2.3 ± 0.2*
Clinical chemistry						
Creatinine	120.2 ± 4.5	111.0 ± 5.6	110.8 ± 11.8	94.0 ± 14.3	97.0 ± 3.7	85.6 ± 7.9
Troponin 1	0.04 ± 0.10	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.10 ± 0.07

Abbreviations: degree (1–5), 1=minimal (minor lesion) and 5=severe (extensive lesion); RBC, red blood cell; WBC, white blood cell.
Data expressed as mean ± s.e.m.
*$P<0.05$ compared to control.
#$P<0.05$ compared to V-Focus low dose (1×10^{10} d.r.p.).

Clinical pathology. Clinical pathology data are shown in Table 2. There were no effects of delivery of AAV2/1SERCA2a on red and white blood cell counts at either 4 or 6 weeks follow-up after delivery ($P>0.05$; red blood cell, white blood cell and lymphocytes). In the 4-week follow-up cohort there was a significant increase in lymphocytes in the recirculation low-dose group ($P<0.05$) compared to both control and recirculation medium-dose animals. However, there was significantly less lymphocytes in both the treated groups ($P<0.05$) compared to controls in the 6-week follow-up cohort. Clinical chemistry parameters measured (Table 2, creatinine and troponin 1) were not significantly altered by delivery of AAV2/1SERCA2a in either the 4- or 6-week follow-up cohorts compared to control groups ($P>0.05$).

Vector persistence. A total of 4 weeks after the administration of AAV2/1SERCA2a at 1×10^{10} d.r.p. (recirculation low dose) qPCR analysis did not detect any signal in the myocardium. Six animals receiving 1×10^{12} d.r.p. AAV2/1SERCA2a (recirculation medium dose) showed a detectable, but not quantifiable vector genome presence (60%, $P<0.05$ vs control, rank sum test). A total of 6 weeks after the administration of 1×10^{13} d.r.p. AAV2/1SERCA2a (recirculation high dose) all seven animals had detectable levels of the vector (100%, 2649 ± 1225 copies per μg DNA, $P<0.05$ vs control), although in one animal the level was not quantifiable. Although a 2.5-fold higher dose of AAV2/1SERCA2a (intra-coronary group) was delivered using intra-coronary infusion qPCR only detected quantifiable levels of AAV2/1 in three of six animals (50%, 1651 ± 872 copies per μg DNA, $P>0.05$ vs control, rank sum test) 6 weeks after delivery, the remaining three animals had undetectable levels of the vector, one animal was excluded as a statistical outlier. Samples from both the liver and the lung were also analysed for vector persistence, although there was a trend for increased vector particles in both the liver (148 773 ± 69 493 copies per μg DNA) and lung (7781 ± 3143 copies per μg of DNA) of intra-coronary group of animals after 6 weeks follow-up there was no significant difference compared to the high-dose V-Focus group (liver 69 595 ± 22 307 copies per μg of DNA; lung 2574 ± 627 copies per μg of DNA; $P>0.05$). AAV2/1 was not detected in tissue from animals in the control groups.

Discussion

In the current study we examined the consequences of increasing the expression of SERCA in a large animal experimental model of HF using a unique recirculation-based method of cardiac gene delivery. Our study was predicated on prior evidence supporting the notion that increased expression of SERCA improves myocardial performance in small animal models of HF. In particular we addressed issues of dosing and delivery mode in a 'pre-clinical' model of HF. The delivery of SERCA led to a dose-dependant improvement in contractility in animals with pacing-induced HF, despite a further 4 weeks of continuous pacing after administration. Myocardial SERCA expression has been increased using gene therapy-based approaches in a number of small animal models, with varying success.[4,8,11] In conjunction with the improvement in contractile function, some deleterious aspects of increased calcium cycling have been observed,[12] such as impaired myocyte shortening at high SERCA1a activity, indicating the potential importance of dose optimization. Our study supports the notion of a beneficial effect of augmented SERCA expression in the failing heart. We did not observe any deleterious effects of altered expression of this key Ca^{2+} handling protein

within the range of the doses examined, such as ventricular arrhythmia or sudden death.

In order to achieve optimal gene delivery to the myocardium, many different approaches have been used. Because of the relatively poor uptake of naked plasmid DNA, emphasis has been particularly placed on the utilization of viral vectors, initially adenovirus and more recently adeno-associated viruses. In the current study we used a well-characterized engineered adeno-associated virus serotype, AAV2/1. Packaging the gene of interest into the AAV-2 results in a 100-fold increased ratio of cardiac to hepatic activity,[13] although serotype 1 has been shown to have limited cardiac specificity.[14] Others have recently compared the cardiac specificity of AAV2/1, AAV2/8 and AAV2/9 and found that intravenous administration of AAV2/9 resulted in a 200-fold greater cardiac expression than AAV2/1[5] and that AAV9 is superior to AAV8 for cardiac specificity when delivered systemically.[15] Although these improvements in our understanding of vector biology have occurred and cardiac specificity of AAV can be increased, these vectors are still able to transduce other tissues when administered intravenously.[5] Therefore, in combination with improved vector specificity, targeted delivery needs to be incorporated to limit systemic effects.

Studies have shown that the optimal expression of AAV occurs at 4 weeks after administration of the agent,[16] although the optimal time for expression of AAV2/1 in the heart has not been described. Consequently, follow-up after administration was extended to 6 weeks to determine the haemodynamic effects of a longer expression time. A positive effect of AAV2/1SERCA2a delivered by cardiac recirculation (V-Focus) was demonstrable despite continued right ventricle (RV) pacing, indicating both the safety of delivering high doses of AAV2/1 (10^{13} d.r.p.) and the chronic benefits of increased SERCA2a expression. Unlike some other studies of cardiac gene delivery,[16,17] we did not observe evidence of myocardial inflammation. This may be related to the lower immunogenicity of AAV2/1, shorter administration time (10 min) and perhaps the result of less systemic delivery.

Despite using a vector of modest cardiac specificity,[5,14] we were able to achieve sufficient gene expression as required to augment ventricular function via cardiac recirculation, although a 2.5-fold higher concentration delivery of AAV2/1SERCA2a by intra-coronary infusion was ineffective in enhancing ventricular contractility. Although, a significant functional effect of recirculating AAV2/1SERCA2a delivery was observed, tissue analysis suggested that low copy number levels of the *SERCA2a* gene were likely present. Nevertheless, the positive functional effect of AAV2/1SERCA is entirely consistent with previous work that shows *SERCA2a* is a low-abundance gene and that only one to two functional copies are required per cell to maintain normal levels of SERCA mRNA and protein.[18] Percutaneous coronary infusion of adeno-viral vectors without recirculation have also shown low-transfer efficiency.[18] The basis of the limited success of intra-coronary delivery is almost certainly a consequence of the transient nature of the method, limiting vector–tissue contact, and faster systemic distribution.[6] Factors that can effect viral gene delivery include coronary flow, vector concentration and endothelial permeability,[19,20] in the case of the V-Focus system there is increased control over the former two and the addition of a cellular permeability agent[21,22] into the system is an easy incorporation. Hayase *et al*. have also examined a percutaneous method of vector delivery to enhance the time frame for more efficient gene transfer.[23] This method requires occlusion of the coronary vasculature for 3 min, allowing the vector of interest to dwell, resulting in improved protein expression. Another method requiring occlusion of the left anterior descending coronary artery (LAD) has been recently tested by Raake *et al*.[24] Selective pressure-regulated retro-infusion was used to deliver AAV vectors with success following a 20 min non-continuous retro-infusion into the anterior inter-ventricular vein. In comparison to these two methods, V-Focus infusion is constant and does not require coronary vasculature occlusion.

An additional major finding in the present study was that optimal augmentation of *SERCA* gene expression resulted in reduction in *BNP* gene expression. BNP is a key marker of the remodelling response of the failing heart and, as such, the reduction in myocardial tissue levels by SERCA gene therapy suggests that this tool may initiate a process of reverse remodelling. The mechanism by which the fall in BNP expressed occurred in the present study was not studied in detail. It has been previously shown that activation of calcineurin, a Ca^{2+}-dependent phosphatase promotes the activation of nuclear factor of activated T cells-mediated hypertrophy including the upregulation of *BNP* gene expression.[25] As such SERCA delivery, and the resultant expected reduction in intracellular Ca^{2+} levels could be expected to lead to a reduction in BNP expression. Although it is acknowledged that the tachy-pacing model may not mimic all facets of the human form of HF, particularly in regard to the potential reversibility of remodelling with the cessation of pacing, we did not stop pacing during this study and control animals demonstrated the typical pattern of progressive remodelling observed in advanced HF.

In conclusion, we have shown that targeted delivery of AAV2/1SERCA using a novel cardiac recirculation system produces chronic and dose-related functional benefit in an ovine model of tachycardia-induced cardiomyopathy. Ongoing development of viral vectors with greater cardiac tropism in conjunction with optimized delivery techniques has the potential for the rapid translation of gene therapy into clinical application for patients with HF.

Materials and methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Initial surgical preparation of animals

A total of 64 adult crossbred sheep were anaesthetized with 2 mg kg^{-1} propofol and maintained on isoflourane and oxygen. A bipolar active fixation lead was placed transvenously in the apex of the RV under fluoroscopic guidance. The proximal end of the lead was connected to a pacemaker reprogrammed for high-rate pacing (Sigma,

Medtronic, Minneapolis, MN, USA) that was positioned subcutaneously in the neck. Terramycin (5 ml, an antibiotic) and Finadyne (1 ml, an analgesic) were administered prophylactically. After 5 days of recovery, RV pacing was initiated at 180 b.p.m.

Protocol

Twenty-nine days after initiation of RV pacing, the pacing rate was reduced to 60 b.p.m. to allow functional measurements to be conducted during the animal's intrinsic rhythm. The animals were anaesthetized using 2 mg kg^{-1} of propofol followed by maintenance i.v. infusion of ketamine (15 mg kg^{-1} h^{-1}) and propofol (30 mg kg^{-1} h^{-1}). Transthoracic echocardiography (iE33 unit; Philips Medical Systems, Bothell, WA, USA) and left ventricular pressure measurements (and subsequent derivatives; Millar Instruments, Houston, TX, USA) were conducted once the animal was stable.

Animals in HF (defined as an ejection fraction between 25 and 35%) were randomized based upon ejection fraction (EF%, Teicholz) into the following groups: control, low-dose AAV2/1SERCA2a (1×10^{10} d.r.p., DNase resistant particles), medium-dose AAV2/1SERCA2a (1×10^{12} d.r.p.) and high-dose AAV2/1SERCA2a (1×10^{13} d.r.p.) via cardiac recirculation (V-Focus; Osprey Inc., Minneapolis, MN, USA), as previously described[10] and intra-coronary delivery (IC, AAV2/1SERCA2a, 2.5×10^{13} d.r.p.). For cardiac recirculation, in brief, perfusate was delivered via a catheter placed in the left main coronary artery and coronary venous blood collected via a purpose-designed balloon catheter placed at the coronary sinus ostium, with concomitant occlusion of the azygous vein. The rationale for the larger concentration of vector in the intra-coronary group of animals was based on a small study in normal animals that indicated that recirculation using the V-Focus system resulted in a 2.5-fold increase in vector uptake (based on real-time PCR methods) compared to direct coronary infusion (data not shown). The control group comprised of a no-dose control group and a control group in which vehicle was administered. There was no treatment effect within these groups and hence the data were combined. A subset of animals in the control group acted as controls for the animals paced 4 weeks post-delivery to examine the dose–response of the agent, the remainder acted as controls for the 6-week paced post-delivery groups for comparison of the recirculation and IC methods.

Following delivery of the vector, all catheters were removed and the animals were allowed to recover. RV pacing was re-initiated the next day and maintained for a further 4 weeks. In the case of the high-dose cardiac recirculation, the intra-coronary and controls pacing was continued for a further 14 days (total 6 weeks pacing post-administration) in an effort to discern the longer term effect of the agent. On the final study day all animals were re-anaesthetized in the same manner as described above and echocardiographic and left ventricular pressure measurements were conducted. Blood sampling was also performed for analysis of clinical pathology. The animals were euthanized using potassium chloride (30 mg kg^{-1} i.v.) and tissues collected for PCR (vector persistence, myocardial BNP and huSERCA expression) and histopathological examination.

Vector and vector delivery

The AAV2/1SERCA2a vector was produced under GLP conditions at Targeted Genetics, Seattle, WA, USA. More specifically, the AAV2/1 viral particle was constructed as a hybrid or pseudotyped vector in which the vector genome incorporates the capsid sequence from AAV1 and the inverted terminal repeat (ITRs) from native AAV2. In conjunction the vector incorporates the human SERCA2a expression cassette under the control of the Cytomegalovirus enhancer/promoter (CMVie).

AAV2/1SERCA was delivered to the heart using one of the two methods. Recirculating (V-Focus) delivery was performed for 10 min as described above, or alternatively intra-coronary infusion was performed by delivering the material (in 20 ml of blood) via constant infusion over 10 min using a standard coronary catheter placed in the left main coronary artery. Pilot studies investigating the expression of the protein in cardiac tissue indicate peak expression at 4 weeks, as a result, follow-up following administration of the vector was at 4 and 6 weeks.

Histopathology and clinical pathology

Tissue samples were formalin fixed and embedded in paraffin, slides were cut and stained using haematoxylin and eosin stain and Masson's trichrome by Experimental Pathology Laboratories Inc. (EPL, Herndon, VA, USA) to determine the degree, if any, of fibrosis formation and chronic myocardial inflammation. Individual findings were graded by blinded examiners on a scale of 1–5 where 1 = minimal (minor lesion) and 5 = severe (extensive lesion). Blood samples taken from animals at the end of the study period underwent haematological and clinical chemistry analysis (Gribbles Veterinary Pathology, Clayton, Victoria, Australia).

Molecular biology

A quantitative PCR reaction (qPCR) conducted by Althea Technologies (San Diego, CA, USA) was used to detect and quantify myocardial abundance of the vector genome of AAV2/1SERCA2a in the left ventricle. The assay detects a 107 bp sequence unique to AAV2/1SERCA2a using the ABI Prism 7700 Sequence Detection System. The number of copies of AAV2/1SERCA2a detected in 1 μg of genomic DNA extracted from each tissue was quantified using serial dilutions of a plasmid containing the target sequences as standards. The lower limit detection of the assay was 20 copies per μg DNA, the lower limit of quantification was 200 copies per μg DNA.

In association, in the animals followed for 6 weeks post-administration of AAV2/1SERCA2a, myocardial BNP and SERCA mRNA expression was evaluated. Total RNA was extracted from left ventricular tissue and reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). BNP and hSERCA mRNA expression levels were analysed by real-time PCR using the primers for sheep BNP (forward: 5′-TGAGCGCCTATCCGCATT-3′ and reverse: 5′-GAGACATCGGACCCAGAAGTG-3′) and SERCA (forward: 5′-GAAATGTCTAACGCCCTCAA CA-3′ and reverse: 5′-GGGCATCCTCAGCAAGGA-3′) and their abundance referenced relative to the housekeeping gene GAPDH (forward: 5-GCCATCACGCCA CAGCTT-3′ and reverse: 5′-GCATCGTGCAGGGACT TATGA-3′) using an ABI Prism 7700 sequence detection

system (Applied Biosystems). Data are presented in ΔC_t format, with subsequent expression as fold difference.

Statistical analysis

Analysis of variance was used to determine differences between pairs of treatment groups where the data represent the change from pre- to post-randomization to each group. Data were compared between the control group, low-dose V-Focus and medium-dose V-Focus and between control group, high-dose V-Focus and intracoronary delivery. All data are expressed as mean ± s.e.m. with a *P*-value of 0.05 considered significant.

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Disclosure

Dr John M Power and Dr David M Kaye are inventors of the V-Focus cardiac recirculation system. Dr Roger Hajjar is a co-founder of Celladon Corporation.

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