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Ameliorated stress related proteins are associated with improved cardiac function by sarcoplasmic reticulum calcium ATPase gene transfer in heart failure

Zhi-Qing Fu[1], Xiao-Ying Li[1], Xiao-Chun Lu[1], Ya-Fei Mi[1], Tao Liu[2], Wei-Hua Ye[3]

[1]*First Department of Geriatric Cardiology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, China*
[2]*Department of Geriatric Ultrasound, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, China*
[3]*Department of Cardiac Surgery, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, China*

Abstract

Background Previous studies showed that overexpression of sarco-endoplasmic reticulum calcium ATPase (SERCA2a) in a variety of heart failure (HF) models was associated with greatly enhanced cardiac performance. However, it still undefined the effect of SERCA2a overexpression on the systemic inflammatory response and neuro-hormonal factors. **Methods** A rapid right ventricular pacing model of experimental HF was used in beagles. Then the animals underwent recombinant adeno-associated virus 1 (rAAV1) mediated gene transfection by direct intra-myocardium injection. HF animals were randomized to receive the SERCA2a gene, enhanced green fluorescent protein (control) gene, or equivalent phosphate buffered saline. Thirty days after gene delivery, the cardiac function was evaluated by echocardiographic testing. The protein level of SERCA2a was measured by western blotting. The proteomic analysis of left ventricular (LV) sample was determined using two-dimensional (2-D) gel electrophoresis and MALDI-TOF-MS. The serum levels of the systemic inflammatory and neuro-hormonal factors were assayed using radioimmunoassay kits. **Results** The cardiac function improved after SERCA-2a gene transfer due to the significantly increased SERCA2a protein level. Beagles treated with SERCA2a had significantly decreased serum levels of the inflammatory markers (interleukin-6 and tumor necrosis factor-α) and neuro-hormonal factors (brain natriuretic peptide, endothelin-1 and angiotensin Ⅱ) compared with HF animals. The myocardial proteomic analysis showed that haptoglobin heavy chain, heat shock protein (alpha-crystallin-related, B6) were down-regulated, and galectin-1 was up-regulated in SERCA2a group compared with HF group, companied by up-regulated contractile proteins and NADH dehydrogenase. **Conclusions** These findings demonstrate that regional intramyocardial injections of rAAV1-SERCA2a vectors may improve global LV function, correlating with reverse activation of the systemic inflammatory, excessive neuroendocrine factors and the stress-associated myocardial proteins, suggesting that the beneficial effects of SERCA2a gene transfer may involve the attenuation of stress-associated reaction.

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Keywords: Heart failure; Sarco-endoplasmic reticulum calcium ATPase; Gene transfer; Stress reaction; Neuro-hormonal factors

1 Introduction

Although considerable advances have been made in our understanding of the pathogenesis and treatment of heart failure (HF) over the past two decades, morbidity and mortality remain high. The development of novel treatments for patients with HF therefore remains a major research priority. A growing body of evidence has accumulated concerning the intracellular Ca^{2+} cycling that plays a key role in the development of HF. The cardiac sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) regulates intracellular Ca^{2+}-handling and thus, plays a crucial role in cardiac contraction and relaxation. In human HF patients, as well as animal models with induced HF, SERCA2a activity is known to decline, and SERCA2a protein and mRNA levels are decreased.[1,2]

Against a background of new, rapidly developing therapeutic platforms, such as cell and gene therapies, the strategy to increase SERCA2a levels by transferring the SERCA2a gene to failing hearts has been suggested as a promising therapy.[1,3] Indeed, administration of the SERCA2a gene during experimental animal HF resulted in improved energy metabolism[4] and decreased arrhythmia[5] accompanied by improved myocardial function. Another study showed that genetic modulation of HF using the SERCA2a gene was associated with improvement in cardiac function and inflammatory markers.[6] Xin *et al*.[7] recently reported that the restoration of SERCA2a expression could attenuate endo-

Correspondence to: Xiao-Ying Li, Professor, First Department of Geriatric Cardiology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, China. E-mail: lixy@mx.cei.gov.cn
Telephone: +86-10-66876274 **Fax:** +86-10-66876274
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plasmic reticulum stress and myocardial apoptosis in an ischemic HF model. However, it still failed to define the effect of SERCA2a overexpression on the systemic inflammatory response and neuro-hormonal factors. The presence of recombinant adeno-associated viral (rAAV) vectors makes it possible due to their low immunogenicity.

The purpose of the current study was to further examine the functional consequences of *in vivo* administration of rAAV mediated SERCA2a gene delivery. Specifically, we hypothesized SERCA2a gene delivery would lead to improvement in myocardial function, and the reversal of negative systemic inflammatory effects. Furthermore, we sought to assess the impact of SERCA2a gene therapy on neuro-hormonal factors and the protein expressions in myocardium.

2 Methods

2.1 Animals

All sixteen male beagles (10–15 kg, 6–8 months of age) provided by the Animal Experiment Center of Chinese PLA General Hospital, were housed with free access to standard food and water. Experimental protocols complied with the Guide for the Care and Use of Laboratory Animal of Chinese PLA General Hospital and were approved by the Chinese Academy of Sciences. All animal experiments were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at our hospital.

2.2 Construction and production of recombinant adeno-associated virus 1 (rAAV1) Vectors

Enhanced green fluorescent protein (EGFP) or human SERCA2a (–18–3486) gene was inserted into vector plasmid (pSNAV) to construct pSNAV/EGFP or pSNAV/SERCA2a. A large scale of rAAV was produced by AGTC Gene Technology Company (Beijing, China) as described.[8] In each vector, gene expression was under control of the cytomegalovirus promoter and the polyadenylation signal provided by simian virus 40. The vector preparations used in this study were diluted to titers of 1×10^{12} vector genomes (v.g.)/mL in phosphate buffered saline (PBS, pH 7.4) and were stored at 4°C.

2.3 Animal experiment and gene delivery

HF induced by rapid right ventricular pacing was performed as described previously.[9] Sixteen beagles were anesthetized with intravenous pentobarbital (25 mg/kg body weight) and a unipolar pacing electrode was inserted through a small incision in the external jugular vein. It was passed to the apex of the right ventricle via the right atrium. A non-synchronous ventricular pulse generator (Fudan University, Shanghai, China) was connected to the lead and inserted in the subcutaneous pocket cranial to the first rib. After three days of recovery, twelve beagles were paced at 230 beats/min (bpm) for four weeks to induce HF while another four beagles were not paced. Animals were observed daily. Congestive HF was established by clinical signs (lethargy, dyspnea and edema) and confirmed by echocardiographic studies [ejection fraction (EF) ≤ 45%].[10] Once HF was established, the pacing rate reduced to 180 bpm to stabilize the condition. Then beagles with HF were randomly divided into three groups (n = 4, each): (1) EGFP group; (2) SERCA2a group; and (3) HF group. Another four unpaced beagles served as control group.

Then intramyocardial gene delivery was used. A left lateral thoracotomy was performed under anesthesia and ventilation (with a tidal volume of 10–15 mL/kg at a frequency of 12–15 bpm). The pericardium was incised. rAAV1-EGFP, rAAV1-SERCA2a (1×10^{12} v.g. per beagle, in 1 mL PBS solution, pH 7.4) and equivalent PBS were injected directly into myocardium with a 20-gauge needle (10 sites for each animal with a 1 cm interval, 5 mm deep) along the anterior left ventricular (LV) wall between the left anterior descending artery and the left circumflex coronary artery. Thirty days after gene delivery, animals were sacrificed by exsanguinations after anesthesia, and hearts were harvested. After trimming away connective tissue and atria, LV tissue was immediately frozen in liquid nitrogen.

2.4 Echocardiographic assessment

To measure cardiac function before the initiation of pacing, echocardiographic assessment was performed with ACUSON Sequoia™ 512 ultrasound system (SIEMENS AG, Germany), on the conscious animal and the pacemaker switched off when stable HF had been established and 30 days after gene delivery. Intraluminal LV dimensions were measured from the trailing edge of the septal wall image to the leading edge of the LV free wall at end-diastole and end-systole. Using B-mode echocardiography, LA chamber size was measured in two dimensions from the point of coaptation of the mitral leaflets to the opposite wall, and in a plane perpendicular to that just described. These values were determined by a single experienced investigator in a blinded fashion. These images and parameters were recorded for subsequent review and analysis.

2.5 Expression of EGFP

Frozen myocardial tissue was mounted in optimum cutting temperature compound and six micrometer serial

sections of the tissue transferred on to slides were sealed with 10% anti-fluorescence quenching glycerol. Each slide contained 3–4 sections representing the heart at different levels. Sections were immediately viewed using a fluorescence microscope.

2.6 Western Blotting

All samples were taken from the injected heart region 30 days after vector administration for protein analysis. To investigate the effects of rAAV1-SERCA2a treatment on myocardial tissue protein expression, proteins were extracted, quantified and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.[11] Blots were incubated with primary antibodies: anti-SERCA2a and anti-β-actin (all from ABcam, UK) which acted as an internal control.

2.7 Measurement of serum concentration of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), endothelin-1 (ET-1), angiotensin Ⅱ(Ang-Ⅱ) and brain natriuretic peptide (BNP)

At the 30th days after gene transfer, a blood sample was collected from each animal and transferred into plastic tubes containing anticoagulant (1: 9, 0.129 mol/L trisodium citrate). The citrated blood was immediately centrifuged at 1,760 *g* for 10 min at room temperature to obtain plasma. The serum TNF-α, IL-6, ET-1, Ang-Ⅱ and BNP concentrations were assayed using radioimmunoassay kits (Radioimmunity Institute, Chinese PLA General Hospital, Beijing, China) according to the procedure described by the manufacturer.

2.8 Proteomics analysis

2.8.1 Protein sample preparation

During protein sample preparation, 100 mg of LV tissue were ground to a fine powder using a liquid nitrogen- cooled mortar and pestle. The powdered tissues were homogenized using a polytron tissue homogenizer (PRO Scientific Inc., USA) in 500 μL lysis buffer composed of 40 mmol/L Tris, 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS (3-[(3-cholamido-propyl)-dimethylammonio]-1-propanesulfonate), 1% DTT (Dithiothreitol), and 1 mmol/L EDTA (Ethylene diamine tetraacetic acid), 20 μg/mL PMSF (Phenylmethanesulfonyl fluoride). After a few cycles of freezing in liquid nitrogen and subsequent thawing, 5 μL deoxyribonuclease (0.01 μg/μL) and 5 μL ribonuclease (0.01 μg/μL) were added. After standing on ice for 20 min, the crude extract was centrifuged at 21,000 *g* at 4ºC for 30 min, and the clarified supernatant was transferred to a new Eppendorf tube and stored on ice. Once the protein concentration was assessed, using Coomassie brilliant blue G250 (CBB-250) with BSA as the standard, the supernatant was used immediately for 2-D analysis or stored at −80ºC until required for later use.

2.8.2 Two-dimensional electrophoresis (2-DE) and image analysis

2-DE and image analysis were performed as previously reported, with minor modifications.[12] In brief, isoelectric focusing (IEF) was carried out on IPGphor (Amersham Biosciences) using precast gel strips. After IEF, strips were equilibrated twice for 15 min in equilibration buffer containing 6 mol/L urea, 30% glycerol (v/v), 2% SDS (w/v) in 50 mmol/L Tris-HCl buffer, pH 6.8, supplemented with: (1) 80 mmol/L DTT for the first treatment and (2) 200 mmol/L iodoacetamide and a trace of bromophenol blue for the second treatment. After equilibration, the IPG strips were placed atop 12.5% SDS-PAGE slab gels and embedded with a 0.5% agarose solution. Gels were run at room temperature in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS. The gels were run at 1,500 mW until the dye front reached the bottom of the gel, fixed in 25% ethanol and 8% acetic acid for 40 min and then stained with CBB-250. The 2-D gels were scanned using a UMAX Powerlook 2,100 XL image scanner (UMAX Inc.). Image analysis and comparative studies were performed using the Image Master 2D Elite V4.01C Image Analysis software (Amersham Biosciences). The procedure was performed by a trained technician who was blinded to treatment allocation. Four gels from the same treatment set (HF or SRECA2a) were used to create an averaged gel. Then the gel spot volumes from SERCA2a averaged gel were compared with the matched gel spot volumes from HF averaged gel as the reference gel.

2.8.3 In-gel trypsin digestion and analysis by mass spectrometry

Protein spots excised from CBB-250 stained gels were digested with trypsin. Briefly, the gel pieces were destained with 100 μL of a 1: 1 mixture of 50 mmol/L ammonium bicarbonate: acetonitrile for 10 min, followed by dehydration in a vacuum centrifuge. Gel pieces were covered with 10 μL digestion buffer, containing 25 mmol/L ammonium bicarbonate and 12.5 ng/μL trypsin. Digestion was allowed to proceed overnight at 37ºC. The resulting peptides were extracted using 5% trifluoroacetic acid (TFA)/50% methyl cyanide for one hour at room temperature, and 2.5% TFA/50% methyl cyanide for another one hour, with the clarified supernatants taken after each respective digestion. The supernatant solutions were combined in an Eppendorf tube and dried down in YZG-600 vacuum concentrator (Changzhou Yutong Drying Equipment CO. LTD. China). MS was performed on a matrix-assisted laser desorption ionization-time of flight-mass spectrometer (MALDI-TOF-MS, Bruker Ulteraflex, Bruker Daltonik, Germany).

2.8.4 Database searching and protein identification

Mass spectral data collected during the MALDI-TOF-MS analysis were processed and converted into a file using the Bruker Daltonics Bio Tools 2.2 software. We used the MASCOT program to identify proteins (Matrix Science, London, UK; http://www.matrixscience.com) against mammalian proteins in the NCBI NR database and accepted proteins as identified if there was a significant MOWSE score ($P < 0.05$), sufficient sequence coverage, correct molecular weight and isoelectric point (pI) value of the corresponding spot on 2-DE. We allowed a maximum of one missed cleavage and a maximum peptide mass tolerance of 0.1 Da for each database search.

2.9 Statistical analysis

Data are expressed as the mean ± SD. Unpaired two-tailed *t*-tests were performed to analyze the statistical differences between the data from control and paced hearts. Comparisons among group means were performed with one-way analysis of variance (ANOVA) followed by Dunnett's test for individual-mean comparisons relative to control. Values of $P < 0.05$ were considered statistically significant.

3 Results

3.1 Evaluation of beagles' heart function

All paced animals (n = 12) developed congestive HF in the 30 days pacing period. Echocardiographic data from these beagles are summarized in Table 1. The average EF was reduced after pacing compared to that before pacing (43.76 ± 6.95 *vs*. 62.93 ± 5.89, $P < 0.05$) and fractional shortening (FS) was also decreased significantantly (21.17 ± 6.30 *vs*. 35.28 ± 5.35, $P < 0.05$). The left heart chamber sizes were enlarged significantly after pacing as measured by LV end diastolic diameter (35.50 ± 2.67 *vs*. 31.46 ± 2.87, after pacing versus before pacing, respectively, $P < 0.05$) and left atrial (LA) dimensions (22.39 ± 2.67 *vs*. 18.56 ± 1.02, after pacing versus before pacing, respectively, $P < 0.05$).

Thirty days after SERCA2a gene transfer, the cardiac function of beagles in SERCA2a group significantly improved. As shown in Table 2, the functional parameters of global LV were increased greatly in SERCA2a group compared with those in HF group, including stroke volume (SV, 24.25 ± 4.65 *vs*. 16.30 ± 2.23, $P < 0.05$), EF (57.87 ± 6.86 *vs*. 45.03 ± 4.97, $P < 0.05$) and FS (29.81 ± 4.55 *vs*. 21.61 ± 2.92, $P < 0.05$). No statistically significant differences in left heart morphologic parameters were observed between the SERCA2a and HF groups.

3.2 Expression of rAAV1-EGFP in myocardial tissue

Thirty days after gene transfer, scattered and dispersed green fluorescence could be observed in the rejected area in the myocardium of the EGFP group animals with the laser scanning confocal microscopy (Figure 1).

Table 1. Summary of the echocardiographic data before and on the 30th day after rapid ventricular pacing.

	LA dimension (mm)	LV diastolic diameter (mm)	Stroke volume (mL)	EF (%)	FS (%)
Before pacing (n=12)	18.56 ± 1.02	31.46 ± 2.87	25.67 ± 3.92	62.93 ± 5.89	35.28 ± 5.35
Thirty days after pacing (n=12)	22.39 ± 2.67*	35.50 ± 2.67*	18.52 ± 3.70*	43.76 ± 6.95*	21.17 ± 6.30*

Data are presented as mean ± SD. *$P < 0.05$ *vs*. control beagles. EF: ejection fraction; FS: fractional shortening; LA: left atrial; LV: left ventricular.

Table 2. The echocardiographic data summary after 30 days with SERCA2a overexpression.

	LA dimension (mm)	LV diastolic diameter (mm)	IVS diameter (mm)	LVPW thickness (mm)	Stroke volume (mL)	EF (%)	FS (%)
Control	17.27 ± 0.87	30.25 ± 3.15	5.95 ± 0.50	5.40 ± 0.48	26.25 ± 4.88	63.69 ± 5.54	34.37 ± 4.25
HF	23.32 ± 2.52*	35.50 ± 1.85*	4.60 ± 0.34*	4.56 ± 0.11*	16.30 ± 2.23*	45.03 ± 4.97*	21.61 ± 2.92*
EGFP	21.97 ± 1.46*	35.55 ± 3.21*	4.55 ± 0.46*	4.61 ± 0.31*	14.50 ± 4.53*	37.84 ± 8.20*	17.68 ± 4.30*
SERCA2a	21.27 ± 1.42	32.77 ± 2.10	5.23 ± 0.27	5.08 ± 0.44	24.25 ± 4.65#	57.87 ± 6.86#	29.81 ± 4.55#

Data are presented as mean ± SD, n=4 in each group. *$P < 0.05$ *vs*. control group, #$P < 0.05$ *vs*. HF group. EF: ejection fraction; EGFP: enhanced green fluorescent protein; FS: fractional shortening; HF: heart failure; IVS diameter: the end-diastolic diameter of the interventricular septum; LA: left atrial; LV: left ventricular; LVPW thickness: the end-diastolic thicknesses of left ventricular posterior wall; SERCA2a: sarcoplasmic reticulum calcium ATPase.

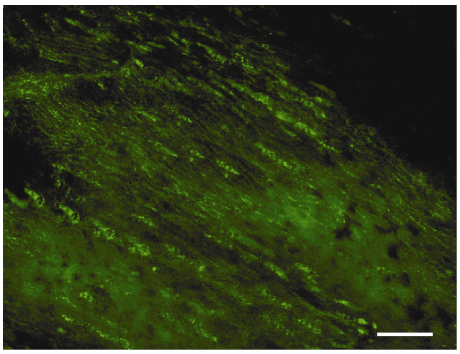

Figure 1. Confocal microscopic image demonstrating expression of green fluorescent protein in left ventricular myocardium of enhanced green fluorescent protein (EGFP) group. The scale bar represents 50 μm.

3.3 SERCA2a protein expression in myocardial tissue

Myocardial protein levels of SERCA2a were demonstrated in Figure 2. SERCA2a levels declined significantly in the HF group compared with those in the control group (protein level/β-actin: 0.70 ± 0.05 *vs*. 1.43 ± 0.03, $P < 0.05$). No statistically significant differences were observed between the EGFP group and the HF group. The SERCA2a-treated animals demonstrated dramatically higher levels of SERCA2a when compared with HF animals (protein level/β-actin: 1.32 ± 0.04 *vs*. 0.70 ± 0.05, $P < 0.05$). This difference was also significant when SERCA2a-treated animals were compared to animals receiving EGFP treatment (protein level/β-actin: 1.32 ± 0.04 *vs*. 0.73 ± 0.03, $P < 0.05$).

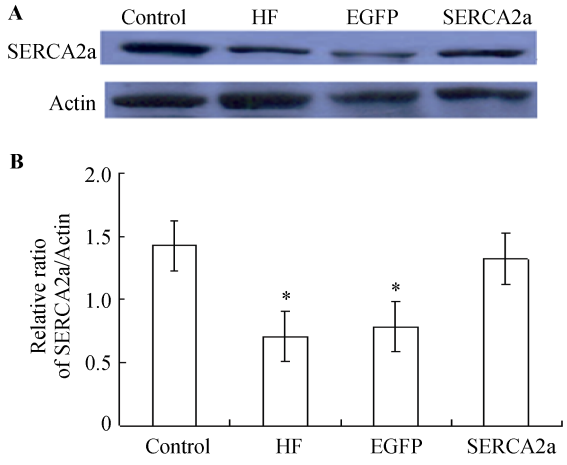

Figure 2. SERCA2a gene therapy increased SERCA2a protein expression in myocardial tissue of HF beagles. (A): SERCA2a protein expression in myocardial tissue of HF beagles was increased after SERCA2a gene therapy; (B): Histogram representing the quantitative analysis of SERCA2a level normalized to actin protein ($n = 4$). *$P < 0.05$ *vs*. HF group. EGFP: enhanced green fluorescent protein; HF: heart failure; SERCA2a: sarcoplasmic reticulum Calcium ATPase.

3.4 SERCA2a overexpression attenuated systemic inflammatory and neuroendocrinal factors

Thirty days after gene transfer, serum concentrations of inflammatory factors TNF-α, IL-6, neuroendocrinal factors ET-1, Ang-Ⅱ, BNP were significantly decreased in SERCA2a group compared with the HF and EGFP groups, and no statistical differences could be found when compared to the control group. However, serum concentrations of the above biomarkers were significantly increased in the HF and EGFP groups, compared to the control group (Table 3).

3.5 2-DE

Computer-assisted spot matching and CBB-250 stained images of the two averaged gels enabled localization of variation spots. Spots detected by CBB-250 staining were excised and subjected to MALDI-TOF-MS for identification. In our experiments, we ran the 2-DE of HF myocardium and overexpressed SERCA2a myocardium (Figure 3). Over 500 protein spots were visualized.

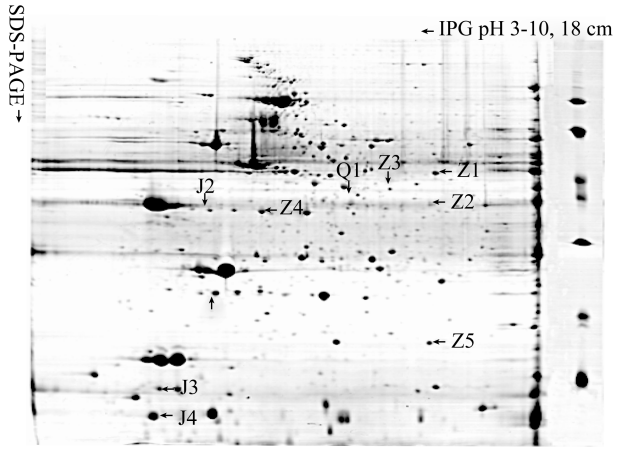

Figure 3. A two-dimensional electrophoresis (2-DE) gel image of beagle left ventricular proteins showing the location of spots significantly altered following SERCA2a overexpression. SERCA2a: sarcoplasmic reticulum Calcium ATPase.

3.6 Protein identification by MALDI-TOF-MS

Of those proteins found to be differentially expressed, we chose 10 protein spots which were significantly increased or decreased in intensity for identification by MALDI-TOF-MS, as shown in Figure 4 and Table 3. Only eight proteins were ultimately identified (Table 4, 5) namely: Myosin light chain 1 (embryonic muscle/atrial isoform), CG30493-PB isoform, Galectin-1, Troponin T (Slow skeletal muscle Troponin T), NADH dehydrogenase 1α subcomplex, Troponin T, haptoglobin heavy chain, and heat shock protein (α-crys

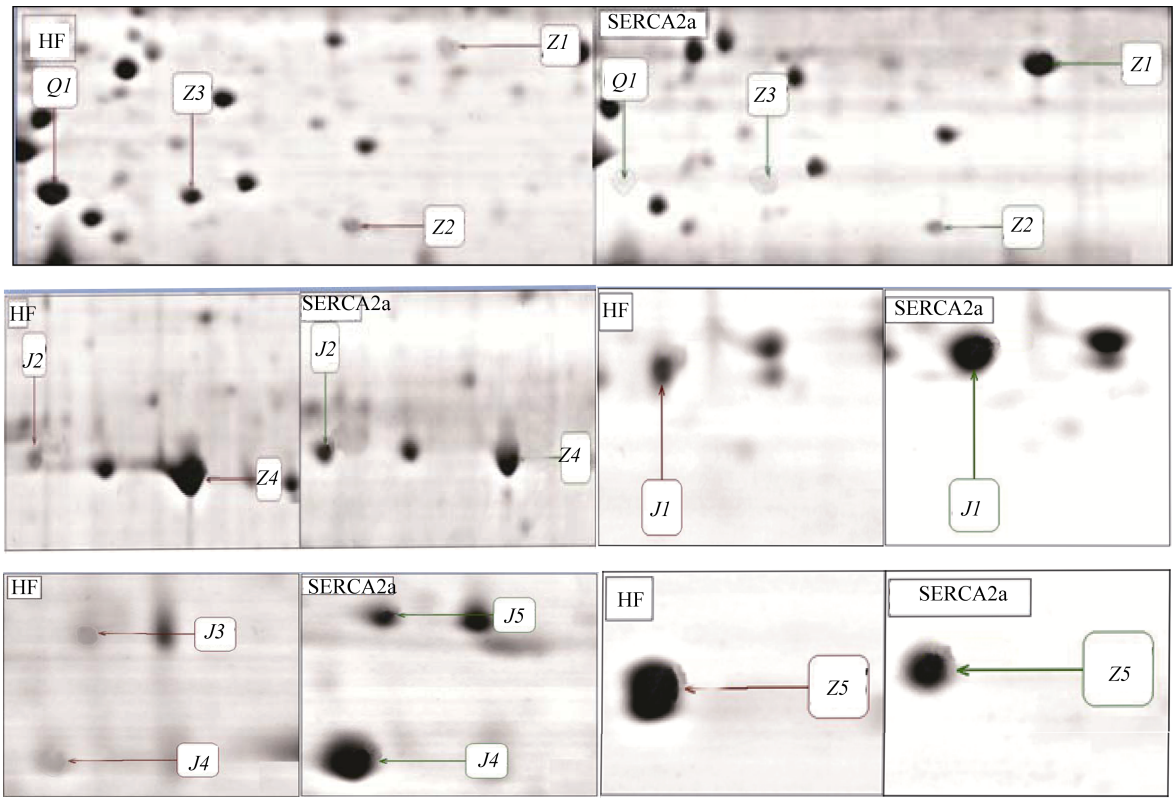

Figure 4. Representative expanded areas of segments of 2D gel map. Arrows indicated ten protein spots which were significantly affected by the experimental protocol (up-regulated: J1–4, Z1–2; down-regulated: Q1, Z3–5).

Table 3. Serum concentrations of BNP, ET-1, Ang-Ⅱ, TNF-α and IL-6 of each group 30 days after gene transfer.

	TNF-α (pg/mL)	IL-6 (ng/mL)	BNP (pg/mL)	ET-1 (pg/mL)	Ang-Ⅱ (ng/mL)
Control (n = 4)	373.08 ± 30.54	93.69 ± 8.57	46.78 ± 29.01	101.90 ± 1.58	160.24 ± 23.61
HF (n = 4)	680.81 ± 100.04*	122.12 ± 20.05*	403.19 ± 247.82*	184.21 ± 58.25*	704.04 ± 187.35*
EGFP (n = 4)	702.65 ± 140.33*	123.80 ± 8.43*	397.54 ± 125.09*	189.34 ± 54.67*	689.36 ± 253.32*
SERCA2a (n = 4)	360.53 ± 81.67$^{\#}$	104.83 ± 8.97$^{\#}$	144.43 ± 58.22$^{\#}$	113.79 ± 37.67$^{\#}$	266.80 ± 173.15$^{\#}$

Data are presented as mean ± SD, n=4 in each group. *P < 0.05 *vs*. control group; $^{\#}P$ < 0.05 *vs*. HF group. Ang-Ⅱ: angiotensin Ⅱ; BNP: brain natriuretic peptide; EGFP: enhanced green fluorescent protein; ET-1: endothelin-1; HF: heart failure; IL-6: interleukin; SERCA2a: sarcoplasmic reticulum calcium ATPase; TNF-α: tumor necrosis factor α.

Table 4. Identified proteins changed in SERCA2a overexpression myocardium.

Spot No.[a]	Accession number	Identified protein	Cover (%)[b]	pI[c]	Mr[c]
J1	gi|57091013	Myosin light chain 1; Embryonic muscle/ Iso-Atrial	74	4.97	21159
J2	gi|73949780	CG30493-PB isoform 1	-	-	35281
J4	gi|73969718	Galectin-1	65	4.83	15122
Q1	gi|73947490	Troponin T (Slow skeletal muscle Troponin T)	45	9.39	28859
Z1	gi|73994237	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	68	6.61	41093
Z2	gi|203656	Troponin T	-	-	35679
Z4	gi|258499	Haptoglobin heavy chain	55	5.80	27269
Z5	gi|57038366	HSPB6	87	5.95	17382

a: Number of the protein spot in the 2-DE gel; b: The sequence coverage of this protein with the matching peptides was indicated; c: Theoretical Mr(Da)/pI were based on the amino acid sequence of the identified proteins and calculated with the Ex-PASy program. HSPB6: heat shock protein (alpha-crystallin-related, B6); pI: isoelectric point; SERCA2a: sarcoplasmic reticulum calcium ATPase.

Table 5. Summary of eight proteins.

Identified protein	Vol. ratio: S/HF[a]	Function
Myosin light chain 1, embryonic muscle/ atrial isoform	2.157	Plays an important role in myocardium contractile activity
CG30493-PB isoform 1	2.038	Unspecified
Galectin-1	6.666	Involved in autoimmune response
Troponin T (Slow skeletal muscle troponin T)	Absent from S	Regulates the strength and velocity of myocardium contractile activity
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	5.730	Increases the synthesis of ATP
Troponin T	2.107	Regulates the strength and velocity of myocardium contractile activity
Haptoglobin heavy chain	0.4607	Acute phase protein, involved in microcirculation stabilization
HSPB6	0.3129	Affects the structure of the cytoskeleton and reinforces the insistence to stress reaction

a: Volume ratio of the regulated proteins based on two independent experimental groups. HF: heart failure myocardium; HSPB6: heat shock protein (alpha-crystallin-related, B6); S: SERCA2a overexpression myocardium; SERCA2a: sarcoplasmic reticulum Calcium ATPase.

tallin-related). Five proteins were overexpressed: myosin light chain 1 (embryonic muscle/atrial iso), CG30493-PB isoform, Galectin-1, NADH dehydrogenase 1α subcomplex and Troponin T, while three proteins were down-regulated: Troponin T (Slow skeletal muscle Troponin T), haptoglobin heavy chain and heat shock protein (α-crystallin-related). The identified proteins were involved in various aspects of myocardial cellular function, including cardiac contraction, metabolism/energetics and reaction to stress.

4 Discussion

Previous studies have demonstrated SERCA2a-based genetic manipulation is associated with improved contractility during HF in failing human myocytes,[13] and *in vivo* big animal HF.[14] These initial results have supported its potential use in the clinical management of HF. To our knowledge, however, investigations have not previously been performed to determine systemic inflammatory and neuroendocrinal effects following rAAV1-SERCA2a gene therapy in rapid pacing induced HF. Our goal in performing this study was to evaluate these specific aspects of SERCA2a-based gene therapy in a big animal model with rapid pacing induced HF.

Several findings are reported in the present study. First, regional intramyocardial injections of rAAV1-SERCA2a vectors may improve LV function, as measured by echocardiographic testing. Second, SERCA2a gene transfer during HF reduced several markers of systemic inflammation and excessive neuroendocrine reaction. Following delivery of the SERCA2a gene, we observed small improvements insystemic levels of IL-6, TNF-α, as well as BNP, ET-1 and Ang-Ⅱ. Third, SERCA2a gene transfer during HF also slightly improved the expression of myocardial stress-associated proteins: haptoglobin heavy chain, HSPB6 (heat shock protein, alpha-crystallin-related, B6), and galectin-1.

The rAAV1 was used as gene transfer vector in the present study, which is one of the most valuable cardiac gene transfer agents available today. Its transfection efficiency was verified in a number of experiments.[15] There are a variety of routes that may be employed to physically introduce rAAV1 to the heart. They are: (1) through the superior vena cava; (2) through the coronary artery; or (3) intramyocardial delivery. In the present study, we used beagle models with global HF induced by rapid pacing. Options for global cardiac delivery include an assortment of intra-venous or intra-arterial routes as well as multiple intramuscular cardiac injections. The two former required sophisticated technicians and costly materials, therefore, we chose the last one, transgene delivery through direct intramyocardial injections from the outside of the heart with a needle. At the same time, the beagles are of a size more suited for surgical manipulations. Thirty days after gene transfer, scattered and dispersed green fluorescence could be observed in the rejected area in the myocardium of the EGFP group animals with the laser scanning confocal microscopy, indicating that direct intramyocardial injections was an efficient method for gene transfer to the myocardium.

Myocardial functional benefits of restoration of the SERCA2a gene have previously been demonstrated by *in vitro* experiments with failing human myocytes,[13] as well as *in vivo* animal experiments of cardiomyopathy.[14] In this *in vivo* model of rapid pacing induced HF, we similarly found that regional multiple intramyocardial injections of rAAV1-SERCA2a improved LV function associated with increased expression of SERCA2a protein. Myocardial proteomics analysis also found that the expression of some contractile proteins and energy production protein were increased in the SERCA2a group, which may be the basis of cardiac function improvement. The changes in the contractile and energy production proteins have been discussed in previous articles,[16–19] and will not be summarized here. The echocardiographic parameters about the left heart chambers were on the down trend, however, they were still not enough to provide the evidence of reverse remodeling in our study.

Therelatively short observing period may be responsible for this result. It is unknown whether administration of SERCA2a results in any detectable significant morphological changes. Histological examination of myocardium and measurement of matrix-regulating proteins may provide more insight into these changes.

A link between increased levels of circulating systemic cytokines and congestive HF is well established.[20–22] We found that our model of HF induced by rapid pacing was associated with a rise in serum levels of potent proinflammatory agents (IL-6 and TNF-α) and excessive neuroendocrine factors (ET-1, Ang-Ⅱ, and BNP). Administration of the SERCA2a transgene during the course of HF resulted in a decreased level of each of these cytokines. According to some previous studies,[23] cytokine activation in the heart may affect SERCA2a expression impairing the ability of the heart to contract. On the other hand, our present study showed that restoration of SERCA2a also suppresses the vicious circles of cytokines, which may result from the alleviated endoplasmic reticulum and mitochondria stress. Further studies are necessary to demonstrate the complicated signal transduction pathways. There were no significant differences in the increased cytokine levels between the EGFP group and the HF group, which may suggest that adeno-associated viral treatment does not increase systemic inflammation.

An important finding in this study was that animals receiving the SERCA2a gene were found to have decreased expression of the stress related proteins (HSPB6 and haptoglobin heavy chain) in the ventricle. It is well documented that HSPB6 plays a vital role in structurally stabilizing the cytoskeleton and reinforcing the insistence to stress reaction.[24] Haptoglobin is also an acute phase protein involved in microcirculation stabilization.[25] According to these facts, we speculate that SERCA2a overexpression gradually relieves the impairment to cardiac myocytes caused by certain stress factors, such as calcium ion influx into the cytoplasm and super-active extracellular neuroendocrine factors. As a result, these stress-associated proteins are down-regulated through a series of signal transduction pathways. Recent evidence showed that restoration of SERCA2a significantly attenuated the endoplasmic reticulum stress and stress–associated apoptosis pathways in the ischemic myocardium. So we speculated that restoration of SERCA2a might attenuate systemic stress reaction through alleviation of endoplasmic reticulum stress, and even mitochondria stress by reducing the calcium overload. On the other hand, galectin-1, which is involved in autoimmune responses, is up-regulated 6-fold within the overexpressed SERCA2a heart. Since galectin-1 has also been shown to induce activated T-cell apoptosis,[26] one might also speculate that the increased expression of galectin-1 in beagles cardiomyocytes could mediate apoptosis as an homeostatic mechanism to shut off autoreactive T cells. Cutting down the cellular immunity may be another pathway to attenuate the systemic stress reaction by restoration of SERCA2a.

Several additional limitations of this study are worthy of mention. We did not study cellular function in regard to calcium transport, but this factor would provide definitive evidence that SERCA2a gene therapy improves myocardial function and stress reaction at the cellular level. Further study will be necessary to assess the changes in the endoplasmic reticulum stress, even the mitochondria stress by reducing the calcium overload. This study used relatively short observation duration (about four weeks). The target gene expression induced by rAAV may sustain a longer period than six months. If we extend the observation period longer, the morphological change of heart chambers may be further understood. Pathologic examination of myocardium may provide evidence on the microscopic structure whether remodeling is reversed. Additional factors to consider in assessing the clinical applicability include the possibility of increased frequency of administration and concomitant immunoreaction. Less invasive means of intracoronary delivery, such as percutaneous or thoracoscopic injection may also further enhance prospects for clinical use.

In summary, the results of this study demonstrate successful rAAV1-based genetic recovery of progressive myocardial dysfunction in a beagle model of rapid pacing induced HF. Genetic modulation of HF using the SERCA2a gene was associated with improvement in myocardial function, attenuating markers of systemic inflammation, excessive neuroendocrine reaction and stress related myocardial proteins.

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