Gene Delivery of Sarcoplasmic Reticulum Calcium ATPase Inhibits Ventricular Remodeling in Ischemic Mitral Regurgitation

Running Title: Beeri et al: SERCA2a Inhibits Remodeling in Ischemic MR

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ABSTRACT

Background: Mitral regurgitation (MR) doubles mortality following myocardial infarction (MI). We have demonstrated that MR worsens remodeling after MI, and that early correction reverses remodeling. SERCA2a is downregulated in this process. We hypothesized that upregulating SERCA2a may inhibit remodeling in a surgical model of apical MI (no intrinsic MR) with independent MR-type flow.

Methods and Results: In 12 sheep, percutaneous gene delivery was performed using a validated protocol to perfuse both LAD and circumflex coronary arteries with occlusion of venous drainage. We administered adeno-associated virus 6 (AAV6) carrying SERCA2a under CMV promoter control in 6 sheep, and a reporter gene in 6 controls. After 2 weeks, standardized apical MI was created, and a shunt implanted between the LV and LA, producing regurgitant fractions of ~30%. Animals were compared at baseline, 1 and 3 months using 3D echo, Millar hemodynamics and biopsies. The SERCA2a group had well-maintained preload-recruitable stroke work at 3 months (decrease by 8±10% vs. 42±12% with reporter gene controls (p<0.001)). LV dP/dt followed the same pattern (no change vs. 55% decrease, p<0.001). LVESV was lower with SERCA2a (82.6±9.6 vs 99.4±9.7 ml, p=0.03); LVEDV, reflecting volume overload, was not significantly different (127.8±6.2 vs 134.3±9.4 ml). SERCA2a sheep showed 15% rise in anti-apoptotic pAkt vs. 30% reduction with reporter gene (P<0.001). Pro-hypertrophic activated STAT3 was also 41% higher with SERCA2a than in controls (p<0.001). Pro-apoptotic activated caspase-3 rose over 5-fold over 1 month in both SERCA2a and controls (p=NS), and decreased by 19% at 3 months, remaining elevated in both groups.

Conclusions: In this controlled model, upregulating SERCA2a induces better function and lesser remodeling, with improved contractility, smaller volume and activation of pro-hyper-trophic/anti-apoptotic pathways. Although caspase-3 remains activated in both arms, SERCA2a sheep had increased molecular anti-remodeling "tone". We therefore conclude that upregulating SERCA2a inhibits MR-induced post-MI remodeling in this model, and thus may constitute a useful approach to reduce the vicious cycle of remodeling in ischemic MR.

Key Words: mitral regurgitation, valvular heart disease, echocardiography, remodeling
Expansion of infarcted tissue begins acutely after myocardial infarction (MI), but a more gradual remodeling process also involves noninfarcted areas,\(^1\) initially compensatory, this process becomes maladaptive, as the ventricle enlarges and contracts poorly\(^2\) with reduced survival.\(^3\)

MI also causes ischemic mitral regurgitation (MR) by altering ventricular geometry and function,\(^4,\,5\) doubling the risk of death. Severe non-ischemic MR has been shown to promote LV remodeling and reduce survival.\(^6\,\,8\) We have previously demonstrated \(^9\) that moderate MR, simulated by an LV-to-LA shunt, added to a small antero-apical MI (causing no intrinsic MR) causes greater ventricular remodeling than a comparable infarction alone, with an earlier transition to a failure phenotype. We have also shown that repairing the regurgitant-type flow at an early stage after the MI reverses the remodeling-related processes.\(^10\) Whole-heart changes parallel cellular and molecular abnormalities in the non-infarcted myocardium that reflect the complex remodeling process. These molecular events also progress differently with MR than with comparable infarction alone, with an initial rise in pro-hypertrophic and anti-apoptotic signals followed by their exhaustion.

Most experimental models of post-MI remodeling use infero-posterior MIs,\(^11,\,12\) but this necessarily links the MI-induced remodeling to the development of MR. The shunt model allows MR to be varied independently in the presence of MI and without interventions such as infarct patching\(^13\) that might themselves influence remodeling.

Upregulating genes encoding for proteins of interest has been demonstrated to be an effective approach to modulate and treat heart failure. One candidate for such gene therapy is the sarcoplasmic (SR) Ca\(^{2+}\)-ATPase (SERCA2a), which is down-regulated in that model, and plays a pivotal role in the regulation of intracellular Ca\(^{2+}\) in cardiomyocytes\(^14\). Calcium entry into the cytosol during systole induces Ca\(^{2+}\) release from the SR through the ryanodine receptor, coupling excitation and contraction. During
relaxation, Ca^{2+} is returned to the SR by the SR Ca^{2+}-ATPase (SERCA2a). Some is also extruded by the sarcolemmal Na^+/Ca^{2+} exchanger (NCX), which is upregulated in cardiac hypertrophy and failure.\textsuperscript{15, 16} Because SERCA2a is the major determinant of the amount of Ca^{2+} available to be released during the upcoming systole, changes in SERCA function significantly affect cardiac excitation-contraction coupling. SERCA2a activity also has a major influence on myocardial relaxation,\textsuperscript{17, 18} Ca^{2+} extrusion via SERCA2a being more efficient energetically than the alternative NCX pathway.\textsuperscript{19} SERCA2a mRNA levels are reduced in failing hearts.\textsuperscript{20} Using a gene therapy approach, up-regulating SERCA2a levels in different models of heart failure resulted in improvement in systolic\textsuperscript{21-23} and diastolic function,\textsuperscript{17} as well as improving metabolism,\textsuperscript{24, 25} potentially reducing arrhythmias\textsuperscript{26, 27} and improving survival.\textsuperscript{25} We have demonstrated that SERCA2A is down-regulated in the remote zones of post-MI remodeling ventricles- significantly more so when MR was also present, accompanied by reduction in contractility of the whole ventricle and of isolated cells, and reduction in single cell calcium transients.\textsuperscript{9} Pathways involved in the compensatory hypertrophic response in remodeling were initially up-regulated, only to fall below baseline at 3 months, when severe dilatation and failure were present. As repairing MR in the early phase, before these processes have been activated, induces reversal of remodeling,\textsuperscript{10} SERCA2a may have a unique role in determining this reversibility. This is emphasized by the recently reported effects of SERCA2a up-regulation in an MR-only pig model of heart failure,\textsuperscript{28} where it induced inhibition of ventricular enlargement and myocardial dysfunction apparent in the control animals.

This study aims to apply the gene therapy approach in a clinically relevant large-animal model of actively evolving remodeling induced by the combination of ischemic and valvular lesions in which a biphasic pattern of compensatory and decompensatory changes has been demon-strated. An intriguing question to address in this model is whether the potentially beneficial effects of SERCA2a gene therapy are accompanied by molecular changes typical of compen-sated hypertrophy\textsuperscript{9} as seen early in the course of MR-augmented remodeling, or only by measurable reductions in ventricular volumes and
improvements in contractile dysfunction without molecular changes in other aspects of the remodeling processes. Genetic modification of such a key pathway can thereby help dissect its contribution to the entire disease process.

Thus, we hypothesized that up-regulating SERCA2a levels by gene delivery using a viral vector may reverse the remodeling process in our model of “ischemic-type” MR, that is, MR associated with myocardial infarction. We also hypothesized that this reversal will be manifest both in ventricular volumes and function, and in persistent activation of pro-hypertrophic and anti-apoptotic pathways.

In this context, prolonged and sustained expression of the transgene is critical, as is the lack of host immune response to the vector. Adeno-associated vector has been demonstrated to confer prolonged and sustained expression of myocardial transgenes, while lacking immuno-génic and cardiotoxic effects, and was therefore used in this study.
METHODS

Animal studies. A total of 12 male Dorsett hybrid sheep (20-30 kg) were included. Our established model of independent MI and MR-type flow\textsuperscript{9,10} was implemented using an 8-cm long, 8-mm diameter reinforced Teflon (PTFE) graft (Edwards, cross-sectional area 0.50 cm\textsuperscript{2}) implanted under sterile conditions into the mid-lateral LV and LA appendage with intramuscular portions stiffened with epoxy resin (Figure 1). The regurgitant flow was confirmed during each thoracotomy using a Transonic flow probe and color Doppler. The standardized shunt diameter and length consistently produced moderate MR (regurgitant fractions of \textasciitilde30\%\textsuperscript{30}). Animals were treated with heparin (3 days) and then oral aspirin.

Vector design: Vector production, harvest, purification, and testing were done as previously described.\textsuperscript{31} The rAAV6.SERCA2a vector used in this study contains an AAV serotype 6 viral capsid and a single-stranded \textasciitilde4.5 kb DNA containing the human SERCA2a cDNA driven by a CMV immediate-early promoter/enhancer, a hybrid intron, and a bovine growth hormone poly-adenylation signal, all flanked by 145 nt AAV2 inverted terminal repeat sequences necessary for replication and packaging of the vector DNA in the capsid. The vector was manufactured using standard calcium phosphate transfection methods in adherent 293 cells. Three plasmids were used, 1 containing helper functions from adenovirus, 1 containing the AAV rep2 and cap1 genes, and the third containing the vector genome. Final vector preparations were more than 95\% pure as judged by SDS-PAGE (Invitrogen, Carlsbad, California).

Gene delivery: Two weeks prior to the first thoracotomy (in order to obtain significant gene expression at model creation), antegrade coronary arterial injection with concomitant great cardiac vein blockade was performed with AAV6 as a vehicle for the reporter gene β-galacto-sidase (β-gal-control) and SERCA2a, each at a titer of $5 \times 10^{14}$ genomes/ml. The great cardiac vein was cannulated via internal jugular access and occluded with a standard balloon-tipped catheter. The left anterior descending
coronary artery (LAD) was cannulated via the femoral artery and occluded with a standard angioplasty balloon before the first diagonal branch. With both arterial and venous balloons transiently inflated for 2 minutes, intracoronary adenosine was administered to increase permeability and prolong dwell time\textsuperscript{32}, followed by $5 \times 10^{12}$ genomes of either AAV6.\textbeta gal or AAV6.SERCA2a (six sheep each). This sequence was repeated for the left circumflex (LCX) artery. Previous work has shown increased tissue expression in the whole adult heart using this delivery method\textsuperscript{33}.

**Model creation:** Sheep were loaded for 3 days with amiodarone (200 mg PO BID), anesthetized with thiopental (0.5 ml/kg), intubated and ventilated at 15 ml/kg with 2\% isoflurane-oxygen, receiving glycopyrrolate (0.4 mg IV) and prophylactic vancomycin (0.5 g IV) and amiodarone (150 mg IV drip). Surface ECG was monitored and a sterile left thoracotomy performed with pericardial cradle creation. A high-fidelity micromanometer-tipped catheter (Millar, Houston, TX) was placed into the LV. After baseline 2D and 3D echo imaging, a septal MI was produced by ligating the mid- to distal left anterior descending coronary artery, known to produce substantial MIs without MR.\textsuperscript{34} 2D echo confirmed that wall motion abnormality involved approximately one-third of the anteroseptum from apex to base for standardization. In addition to analgesia, propranolol, 1 mg IV in two doses, was given for evident stress and tachycardia (>150) upon extubation. Antibiotics (Cephapirin, 0.5 gm IV) and analgesics (Buprenorphine, 0.3 mg BID) were administered for 5 days, and oral amiodarone (200 mg BID) for three.

During repeat sterile thoracotomy at day 30, 3D echo evaluated LV remodeling and function, with directed TruCut needle biopsies of the noninfarcted myocardium near and remote from the border zone. At day 90, 3D echo and blood sampling were repeated at thoracotomy, followed by euthanasia. Animal studies conformed to NIH guidelines (National Research Council, Washington, DC, 1996) and were IRB-approved.
3D echo and LV function. Rotated apical images were obtained at 10-degree intervals with an epicardial 5MHz TEE probe (Sonos 7500, Philips, Andover, MA), rotated by software and gated to ECG and respiration. Digital images were analyzed on a workstation with custom programs, by an operator blinded to treatment assignment. Endocardial surfaces were traced to calculate LV volumes validated against a 36-crystal sonomicrometer array. Remodeling was quantified in terms of increasing LV volumes. Regurgitant fraction was calculated as MR-equivalent flow by Transonic flow-meter, divided by LV ejection volume, with verification of MR flow by pulsed Doppler time-velocity integral of shunt flow multiplied by shunt cross-sectional area. LV pressure-volume loops at the initial and final thoracotomies were obtained using Millar catheters and subendocardial crystals (Sonometrics, London, ON, Canada), with IVC occlusion to obtain pressure-volume curves and derive preload-recruitable stroke work. Crystals were not placed at day 30 to maximize sterility and survival. Maximal systolic dP/dt was obtained by the high-fidelity Millar catheter.

Molecular assays. We measured levels of several molecular species associated with remodeling that modulate cell hypertrophy and death and are responsible for extracellular matrix turnover. All protein assays were performed for each treatment group and stage on each individual sheep separately, and the results were averaged.

Calcium cycle. Sarcoplasmic reticulum (SR) membrane was obtained using sucrose gradient centrifugation. Proteins were separated and an immunoblot using monoclonal anti-SERCA2 and anti-phospholamban (Santa Cruz Biotechnology, Santa Cruz, CA) was performed, normalized to total protein. Na⁺/Ca²⁺ exchanger (NCX) levels were measured by Western blot, using monoclonal anti-NCX antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).
**Pro-hypertrophic and pro-apoptotic cascades.** We measured levels of Akt (protein kinase B) and gp130, which are both at their respective levels (cytosol and membrane) important crossroads in pro-hypertrophic signaling; phosphorylated (activated) STAT3, an important downstream effector of gp130; and activated caspase-3, the final common pathway for intra-cellular apoptosis signaling. Western blot analysis was performed on cell lysates from biopsies at baseline and days 30 and 90. Anti-gp130, anti-phosphoAkt, anti-phospho STAT3 and anti-activated caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA) were detected with peroxidase-conjugated anti-mouse IgG and chemiluminescence, with α-actin as housekeeping control. Integrated blot pixel density was assessed using standard software (ImageJ, NIH) by an operator blinded to treatment assignments.

**Statistics.** All values are reported as mean±SD. Statistical analysis used 2-tailed Student’s t-test for continuous variables compared at specific time-points; the Bonferroni correction was applied when appropriate. Repeated measures over time were analyzed with repeated-measures ANOVA (JMP 8, SAS Institute). P<0.05 was considered significant. Inter- and intra-observer variability for 3D echo-measured LV volumes in our lab were 3.5% as previously reported.9
RESULTS

Infarct size, traced and integrated by 3D echo, was 12-22% of the endocardial surface area, with a mean of 17±3% (n=12).

**Function and volumes:** The SERCA2a group had well-maintained preload-recruitable stroke work at 3 months sacrifice (decrease by 8±10%) vs. a 42±12% decrease with reporter gene controls (p<0.001, Fig. 2). Peak systolic LV dP/dt followed the same pattern (no change vs. 55% decrease, p<0.001, Fig. 2).

Although 3D echo-derived LVEF was decreased in both groups beginning with the post-MI baseline, it was better maintained at sacrifice with SERCA2a (35.2±4.0% vs. 26.1±3.5%, p=0.01, Fig. 2). This was accompanied by a lower LVESV with SERCA2a (82.6±9.6 ml vs 99.4±9.7 ml, p=0.03, Fig. 3); LVEDV, reflecting the volume overload, was not significantly different at sacrifice (127.8±6.2 ml vs 134.3±9.4 ml, p=NS, Fig.3).

Although no quantitative assessment of animal well-being could be performed, the animals in the control group were less active and seemed more short of breath.

**Molecular pathways of remodeling:** As expected, in the SERCA group there was a very significant increase in SERCA2a protein levels in both remote and border zones at sacrifice, which was already apparent at 1 month, follow up, while control sheep demonstrated a sharp reduction in SERCA2a levels at 1 month follow-up (average integrated density 85.6±15.2 vs 54.2±10.8 p<0.001, Fig.4) and even more so at sacrifice (average integrated density 93.6±20.1 vs 34.2±6.3, p<0.001, Fig. 4). Of note, no significant change was noted in regulatory phospholamban levels (Fig.4). NCX levels were significantly more elevated in the control sheep as compared with the SERCA sheep at sacrifice, consistent with a more active remodeling process39 (P=0.025, Fig. 5).
SERCA2a sheep showed at 3 months’ sacrifice a 15% rise in anti-apoptotic phospho-Akt vs. 30% reduction with reporter gene (P<0.001, Fig. 5). The sample mean of STAT3 was also 41% higher at sacrifice with SERCA2a than reporter gene (p<0.001, Fig. 5). In contrast, gp130 fell by 25%-26% in both groups (p=NS by repeated-measures ANOVA), raising the possibility that improved contractility blunted the stimulus for pro-hypertrophic compensation, or alternatively, that SERCA2a over-expression compensates for but does not entirely eliminate the remodeling drive. Pro-apoptotic activated caspase-3 rose over 5-fold and to a comparable extent over 1 month in both SERCA2a and reporter gene animals (p=NS, Fig.5), and decreased by only 19% from 1 to 3 months, remaining elevated in both groups at sacrifice.
DISCUSSION

A large number of patients with MI develop MR and progress to congestive heart failure. Our previous results\(^9\) have shown that, for a comparable infarct size, the presence of MR-type volume over-load leads to greater LV dilatation and dysfunction and to more severe changes at a cellular and molecular level. Molecular changes are biphasic, with initial upregulation and subsequent exhaustion of pro-hypertrophic and anti-apoptotic pathways that otherwise remain elevated when MI is not accompanied by MR. Maintained elevation of caspase-3 and extracellular matrix turnover lead to a failure phenotype with abnormal cellular morphology, decreased calcium cycling, and reduced sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a).\(^9\) This reduction was more pronounced in the border zones of the infarction, reflecting a probable larger element of cell loss through ischemic damages, but was also significant in the remote zones, possibly reflecting stretch-induced activation of the fetal program in these myocytes, resulting in diminution of SERCA2a levels. We have also demonstrated the corollary that early repair of such moderate MR-type volume overload reverses these progressive remodeling processes, and activates intracellular signals promoting hypertrophy, opposing apoptosis, and inhibiting matrix proteolysis.\(^10\)

Manipulating the expression of key proteins and the activity of specific down-stream signaling pathways involved in cardiac hypertrophy and failure will allow us to understand their contribution to the disease process.

AAV (adeno-associated virus) is a gene therapy vector that provides gene expression lasting more than a year in muscle and brain with little or no immune reaction.\(^40-42\) SERCA2a was chosen as transgene because its expression is reduced in our MI+MR model\(^9\), and its over-expression improves contractility\(^21, 23, 43\) and might also decrease apoptosis by reducing intracellular diastolic Ca\(^{2+}\) concentrations.\(^44, 45\) In fact, a phase 1 clinical trial using AAV1.SERCA2a has been completed in
patients with severe heart failure showing safety and positive biological effects (albeit in an open label trial). 

In our model, using a percutaneous delivery system for AAV6 encoding SERCA2a, we managed to secure robust transduction, as manifested by sustained elevation of SERCA2a levels as compared with a significant reduction in controls. We did not detect a compensatory increase in inhibitory phospholamban expression. This up-regulation translated into preserved LV contractility as measured by preload-recruitable stroke work, a relatively load-independent measure of LV function; LV dP/dt was also preserved, while these measurements where significantly depressed in the control animals.

Morphologically, there was less evidence of remodeling in the SERCA animals, manifested as relatively preserved LV end-systolic volumes throughout the experiment. On the other hand, we did not detect a significant change in activated-caspase3 levels - suggesting that while the net tone in the cell is shifted to anti-apoptosis, as demonstrated by Akt and STAT3 activation, upregulating SERCA might not ablate all aspects of the intracellular remodeling cascade. One interesting aspect of the molecular changes was the effect on NCX expression in our model. An increase in NCX expression has been observed in a number of models of heart failure and has been associated with an increased risk of ventricular arrhythmias. In our model, NCX was increased in the control group but was remained at baseline levels with overexpression of SERCA2a. Likewise, we did not detect increased levels of gp130, as we have previously seen with MR repair; however, activated STAT3, downstream from gp130, was significantly increased, suggesting the possibility of greater activation of gp130-containing cytokine receptors, STAT3 activation by an alternative pathway, or decreased feedback inhibition. The results suggest that improving contractility and relaxation is insufficient to reverse the remodeling process completely at a molecular level. Nonetheless, the improvement in contraction, volumes and intracellular pro-hypertrophic pathways suggests that SERCA2a upregulation does at least strongly inhibit the remodeling process. As SERCA2a upregulation has been demonstrated, in different models, to improve
function and retard progression to heart failure, our results are consistent with previously reported data.

This study has several limitations: Ischemic MR affecting a native valve often progressively increases, but is inherently linked to the underlying MI and not standardized. Based on the study motivation, it was critical to separate the two processes of infarction and regurgitation to determine the incremental role of MR and to do so with a standardized orifice, which provided stable regurgitant fractions of ~30% throughout the study. In the clinical situation of the tethered mitral valve, SERCA2a may have an even more pronounced effect by reducing the severity of this dynamic MR: increased LV contractility and decreased LV volumes will increase the closing forces and decrease the tethering forces on the mitral valve, thereby improving coaptation and reducing MR. This will be examined in a separate study. We performed gene delivery 2 weeks before induction of MR+MI. This was done in order to have an established up-regulation of SERCA2a coincident with the initiation of the remodeling process, which starts immediately after infarction, to provide a proof of concept about the role of SERCA2a in this situation. Variations in timing of SERCA2a therapy relative to MR repair will also be assessed, for example, in the fully dilated remodeling state, as there may be a “point of no return” beyond which these interventions may be ineffective.

In conclusion, we have demonstrated that up-regulating SERCA2a in a model of MR+MI may inhibit the remodeling process, as manifested by ventricular function, volumes, and intra-cellular pathways of hypertrophy. This may constitute a potentially useful approach to reduce the vicious cycle of remodeling in ischemic MR.
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DISCLOSURES

None.
REFERENCES


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FIGURE LEGENDS

**Figure 1** - Model of apical MI and independent MR: LV-to-LA shunt (arrows).

**Figure 2** - LV functional parameters: Preload recruitable stroke-work (PRSW), maximal systolic derivative of pressure development (max dP/dT) and 3D echo-derived LV ejection fraction (LVEF). A significantly better global LV function is noted in the SERCA (solid lines) group as compared with controls (dashed lines) (p<0.001 by repeated-measures ANOVA), which is apparent already at 1 month follow-up (*:P<0.001, #:P=0.002, ¶:P=0.003, $:P=0.01).

**Figure 3** - LV end-systolic and end-diastolic volumes from 3D echo analysis. There is a lower end-systolic volume at sacrifice in the SERCA group (solid lines, P=0.001 by repeated-measures ANOVA) as compared with controls (dashed lines) while no difference was found in end-diastolic volumes. (*=P:0.03)

**Figure 4** - Enhanced widespread expression of SERCA2a protein in AAV.SERCA2a (black) sheep at 1 month follow-up and 3 months sacrifice as compared with reduced expression in controls (white). Note that there was no change in phospholamban levels in both groups. (*:p<0.0005 vs baseline, #:p<0.0001 vs SERCA group at 3 months)

**Figure 5** - Levels of pAKT, STAT3, Caspase3, gp130 and Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) at baseline, 1 month follow-up and 3 months sacrifice at the remote zone. Note that pAKT and STAT3 levels are significantly more elevated at sacrifice in the SERCA2a group (solid lines) as compared with controls (dashed lines), while NCX levels are more elevated significantly in the control group (P<0.05 by repeated-measures ANOVA). No significant difference between the groups in caspase3 and gp130 mean levels was detected. (*:P<0.001, #:P<0.03)
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