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Ultrasonic Cavitation-Enabled Treatment for Therapy of Hypertrophic Cardiomyopathy: Proof of Principle

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Abstract

Ultrasound myocardial cavitation enabled treatment (MCET) was applied to the SS-16^{BN} rat model of hypertrophic cardiomyopathy (HCM) for proof of the principle for myocardial reduction therapy. A focused ultrasound transducer was targeted using 10 MHz imaging (10S, GE Vivid 7) to the left ventricular wall of anesthetized rats in a warmed water bath. Pulse bursts of 4 MPa peak rarefactional pressure amplitude were intermittently triggered 1:8 heartbeats during 10 min infusion of a microbubble suspension. Methylprednisolone was given to reduce initial inflammation and Losartan was given to reduce fibrosis in the healing tissue. At 28 d post therapy, MCET significantly reduced the targeted wall thickness by 16.2% ($P < 0.01$) relative to shams, with myocardial strain rate and endocardial displacement reduced by 34% and 29%, respectively, which are sufficient for therapeutic treatment. Premature ECG complexes and plasma troponin measurements were found to identify optimal and suboptimal treatment cohorts and would aid in achieving a desired impact. With clinical translation, MCET treatment should fill the need for a new non-invasive HCM therapy option.

Keywords

Hypertrophic Cardiomyopathy; Myocardial Contrast Echocardiography; Ultrasonic Cavitation; Cardiac Myocyte Necrosis; Cardiac Fibrosis

Introduction

The need for myocardial reduction arises for septal obstructive hypertrophic cardiomyopathy (HCM) and other ventricular hypertrophies, such as apical hypertrophy. HCM is one of the most common genetic cardiovascular diseases, occurring in more than 1 in 500 people (Maron and Salberg, 2014; Semsarian et al. 2015) and puts patients at risk for sudden death (Maron and Maron, 2013). There is a wide range of variation in clinical presentation with treatment tailored to each patient (Enriquez and Goldman, 2014). Hypertrophy leads to

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obstruction of the left ventricular outflow path, either at rest or with provocation, in up to 75% of patients. About one-third of patients with obstruction remain symptomatic after pharmacological therapy and are candidates for myocardial reduction (Marian, 2009).

Hypertrophy can occur in several regions of the myocardium, and is particularly troubling when restrictions occur in the left ventricular outflow tract (LVOT). Apical hypertrophy is also troubling and currently has no available corrective therapy (Jan et al. 2016). Surgical septal myectomy is presently the primary method for reduction of asymmetric septal hypertrophy with symptomatic LVOT (Enriquez and Goldman, 2014; Gersh et al. 2011). Alternatives to surgery have been sought using various technologies. Thermal ablation with high intensity focused ultrasound (Otsuka et al. 2007) or radio-frequency ablation (Lawrenz et al. 2011) have yet to provide a safe alternative. Transcatheter septal ablation with alcohol represents a viable alternative to surgery (Gersh et al. 2011; Leonardi et al. 2010). However, this procedure has relatively high incidence (~ 20%) of heart block requiring permanent pacemaker (Marian, 2009; Singh et al. 2016) and has a substantial risk of serious arrhythmia (Nishimura et al. 2010). The immediate therapeutic effect for alcohol ablation may actually reflect akinesis of the effected region of alcohol ablation with septal thinning of about 24% developing 6 months after treatment (Dabrowski et al. 2012; Baggish et al. 2006). None of these techniques are fully satisfactory, suggesting that an innovative, safe, and effective new therapy for this condition is warranted.

We have developed a novel ultrasonic technique called myocardial cavitation-enabled treatment (MCET), which employs intermittent pulsed ultrasound exposure with peak rarefactional pressure amplitudes about twice the guideline upper limit for diagnostic ultrasound (Miller et al. 2005) of the myocardium together with ultrasound contrast-agent microbubbles to produce cavitation nucleation and tissue injury. Our research thus far has used single element focused transducer with fixed beam, but larger volumes could be treated by beam scanning with a scanning system capable of high pulse amplitudes. This treatment produces scattered nucleation sites of ultrasound-induced injury within the focal zone primarily to cardiac capillaries, with leakage and small hemorrhage, and injury of the adjacent cardiomyocytes for supra-threshold ultrasound pressure amplitudes, leading to micro-scale surgical impacts of local cardiomyocyte necrosis and microscopic lesions. The microlesions accumulate with each intermittent exposure to achieve a desired total impact. In initial animal studies, MCET lesion creation was dose-dependent, and adjustable by manipulation of the ultrasound focus. When triggered at the end of systole, ECG premature complexes (PCs) indicated microlesion occurrence (although arrhythmia could be avoided by triggering at the R wave without loss of efficacy), and the degree of injury correlated with plasma troponin levels (Miller et al. 2014a; Miller et al. 2014b; Miller et al. 2015). Of note, cardiac function was preserved during therapy and no conduction abnormalities, such as ST segment elevation, persisted beyond a few hours (Miller et al. 2014a; Zhu et al. 2015a). Additional studies in normal rats evaluated the acute effect and long-term maturation of the myocardium treated with MCET to assess the potential for therapeutic effect (Lu et al. 2016). These studies revealed significant increases in heart wall thickness a day after therapy, likely secondary to the inflammatory response to the injury. Wall thickness returned to normal four weeks after exposure and pathology revealed significant fibrosis and depletion of myocytes in the treatment zone. These results suggested that MCET might be a

feasible and relatively safe technique to reduce viable myocytes in thickened areas in patients with HCM, although no significant wall reduction was observed. Further studies were necessary to optimize long-term tissue reduction and to limit the initial inflammatory response.

Building on the previous work, this study was designed as a proof of principal evaluation of MCET in a rat model of HCM. The consomic Dahl SS rat with chromosome 16 from brown Norway rats (SS-16^{BN}) undergoes progressive left ventricular (LV) hypertrophy without hypertension (Chao et al. 1998; Klotz et al. 2006; Kriegel and Green, 2008). In this study, SS-16^{BN} rats were treated with MCET to assess acute tolerance of the procedure. In addition, based on work focusing on reduction of fibrosis in myocardial infarction (Roberts et al. 1985; de Carvalho Frimm et al. 1997), steroids and angiotensin II receptor blocker were utilized to decrease the acute inflammation response and decrease fibrosis seen after MCET therapy (Lu et al. 2016). Finally, a chronic test of MCET versus sham was conducted utilizing steroid and anti-fibrotic adjuvants with treatment evaluation by counting PCs and plasma troponin measurement. This study with follow-up for 28 d was conducted to assess the true impact on myocardial function and wall thickness in the treatment zone, and provide the proof-of-principle for myocardial reduction therapy.

Methods

Animal preparation

All *in vivo* animal procedures were conducted with the approval and guidance of the Institutional Animal Care and Use Committee of the University of Michigan, which conform to the NIH guidelines (Guide for the Care and Use of Laboratory Animals). In preliminary studies, Dahl SS rats (Charles River, Wilmington, MA, USA) were used owing to the widespread use of this strain as a model of human disease, including cardiomyopathy. The Dahl SS rats were treated at 8–9 w of age with up to 4 w follow-up. For the main study, SS-16^{BN} rats (Medical College of Wisconsin, Milwaukee WI) were used as a specific model of hypertrophic cardiomyopathy without hypertension (Kriegel and Green, 2008). The veracity of this model for the human condition is only general, but there are few alternatives for a rat model of HCM (Shepard and Semsarian, 2009). Although there was evident hypertrophy in the SS-16^{BN} model, there was no ultrasonically identifiable outflow tract obstruction, nor pathologically identifiable areas of myofiber disarray, which are common features of the human disease. These rats were obtained soon after weaning and housed for up to 13 m before treatment to allow development of the myocardial hypertrophy.

For ultrasound treatment, the rats were anesthetized by intraperitoneal injection of a mixture of ketamine (90 mg kg⁻¹) and xylazine (9 ml kg⁻¹), monitored by observation and pedal reflex. The left thorax was shaved and depilated for ultrasound transmission to the heart. A 24 gauge cannula (BD Angiocath, Becton Dickinson Infusion Therapy Systems, Sandy, UT, USA) was inserted into the tail vein for intravenous injections of contrast agent. The rats were mounted on a positioning board and needle electrodes were placed in the forelegs and left hind leg for detection of the electrocardiogram (ECG). The rat ECG signal was amplified (Model ECGA amplifier, Hugo Sachs Elektronik, Harvard Apparatus, March, Germany), displayed on an oscilloscope for trigger timing (Model TDS 520 B, Tektronix

Inc., Beaverton, OR, USA) and stored for computer analysis of premature complexes (PCs) (Chart Pro 5, ADInstruments Inc., Colorado Springs CO USA). PCs during contrast enhanced ultrasound are indicative of myocardial injury (Miller et al. 2011). The board was then placed in a 38°C water bath filled with degassed water which maintained the rat body temperature and provided coupling for ultrasound scanning and treatment. For follow-up echocardiography or other exams, the rats were anesthetized by inhalation of 1–4 % isoflurane in oxygen (Isotec 4, Surgivet Inc. Waukesha, WI USA), and scanned in the supine position on a warming pad. Each animal was euthanized under anesthesia by heart removal.

Ultrasound

Ultrasound exposure for treatment was provided by a laboratory system with guidance by diagnostic ultrasound imaging, as described previously (Miller et al. 2014a). Briefly, the treatment system consisted of a function generator for generating a pulse train (model 3314A function generator, Hewlett Packard Co., Palo Alto CA, USA), an arbitrary waveform generator for amplitude modulation of the pulse train (model 33220A, Agilent Technologies, Loveland CO, USA), and a power amplifier (A-500, Electronic Navigation Industries, Rochester NY, USA). A 1.5 MHz single element transducer (Panametrics A3464, Olympus, Waltham, MA, USA) with 1.9 cm diameter and 3.8 cm focal length was used for the treatment. The pulse exposure parameters were measured with a calibrated hydrophone (model HMA0200 membrane hydrophone, Onda Corp. Sunnyvale CA USA), and used to set a 4 MPa peak rarefactional pressure amplitude. The –6 dB lateral beam diameter at the focus was measured to be 3.5 mm. The –6 dB axial focal zone was projected to extend from about 25 to 50 mm, which passed through the entire heart (Zhu et al. 2015a). The treatment was targeted in a transverse plane between ribs at approximately the middle of the left ventricle with the aid of diagnostic ultrasound imaging (GE Vivid 7 with 10S probe, General Electric Corp., Cincinnati OH, USA) operated at 10 MHz with 5 cm focal depth, as previously described (Miller et al. 2014a). The imaging probe and therapeutic transducer were clamped together at a 37° angle so that the transducer focus and heart were located in the image during treatment. The function generator created 10-cycle pulses at a 4 kHz pulse repetition frequency, which was modulated to provide bursts of 8 pulses triggered from the ECG at end systole, every 8 heartbeats. Treatments were 10 min in duration.

Ultrasound contrast agent

A continuous infusion of a lipid-stabilized microbubble suspension, developed for use as an ultrasound contrast agent (Unger et al. 1993) and later for therapeutic cavitation nucleation (Unger et al. 2002), was given during treatment. The suspension was prepared to closely duplicate the clinical ultrasound contrast agent Definity® (Lantheus Medical Imaging, Inc., N. Billerica, MA), as described previously (Lu et al, 2016; Miller et al, 2016). A lipid blend was made using powder forms of dipalmitoyl-sn-glycero-3-phosphate (DPPA, Sigma-Aldrich, Milwaukee WI, USA), dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, Alabaster, AL, USA), and dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (DPPE-PEG5000, Avanti Polar Lipids). The lipid blend was sterile filtered using a 0.22 µm filter and then aliquoted into small vials similar to Definity vials. The headspace for each vial was filled with octafluoropropane (HC-218, PurityPlus, Metro Welding Supply, Detroit MI, USA) and the vial was then subsequently

capped and sealed. For use, the vials were shaken exactly as performed for Definity to produce the suspensions of stabilized microbubbles. This agent served as the source of *in vivo* cavitation nuclei for MCET, and contained $3.7 \pm 0.3 \times 10^9$ microbubbles per mL of $1.8 \pm 0.11 \mu\text{m}$ diameter. This was diluted in sterile saline in a 5 mL syringe, and infused via tail vein with a syringe pump at a rate providing $5 \mu\text{L/kg/min}$ of agent.

Steroid and anti-fibrotic therapy

Preliminary tests were conducted to assess the use of methylprednisolone and losartan as adjuvants for MCET treatment to reduce the acute inflammatory response and subsequent scar formation. The preliminary tests and results are described in the Supplemental Material. In this study, 1 mg/kg methylprednisolone was given at therapy and at 3, 6 and 24 hr post treatment, and 10 mg/kg losartan was given daily by gavage starting one day after ultrasound treatment and continuing for 4 wk.

Measured endpoints

Rat weights were measured at treatment and weekly post treatment. Plasma troponin was measured in samples obtained 4 hr after treatment, as a measure of overall cardiomyocyte injury. Plasma troponin I (CTNI-2-HSP, Rat Cardiac Troponin-1 ELISA kit, Life Diagnostics Inc. West Chester, PA USA) is a sensitive measure of cardiac injury in rodents with essentially zero background in controls (O'Brien et al. 2006).

Potentially therapeutic effects on the myocardial wall were measured by echocardiography and by evaluation of histological sections. For the echocardiographic wall measurements the treated region was identified accurately by the region of inflammatory swelling for the early (1 d to 1 w) exams, and by the scarring for the 28 d exams, both of which were evident by qualitative observation of hyper-echogenicity in the ultrasound image, although these regions were different in size for the two time points (see Results). The hyper-echogenicity arises from micro-inhomogeneous layers: for the swelling, these extended over a relatively wide area, while for the scarring, the area was more narrowly confined to the targeted region. These thickness measurements were compared to matched regions in sham treated hearts.

Ejection fraction was measured using the echocardiography features of the diagnostic machine as a general measure of heart function. The potentially therapeutic effect of local reduction in kinetic contraction of the heart wall was measured by echocardiography strain analysis. The 10 MHz images of the heart motion were readily capable of resolving the rat heart wall and were stored and analyzed for pre-treatment (0 d), 1 d, and 28 d post treatment. Image analysis software (EchoInsight, Epsilon Imaging, Ann Arbor MI USA) was used to evaluate radial strain fraction (elasticity) during systole. The strain fraction was measured for an anterior segment 0.25 cm wide enclosing the treated region, as a measure of contractile function. In addition, the displacement of the endocardium during systole was evaluated for the same segment, as a measure of the inward motion, which would potentially be important for HCM at the left ventricular outflow path.

The Evans blue staining was performed as described previously for evaluation of lethally injured cardiomyocytes (Miller et al. 2014a; Miller et al. 2014b). Briefly, Evans blue dye in saline (20 mg/mL) was infused into the animals at 100 mg/kg and hearts were removed the

day after exposure and cleared of most blood with heparinized saline. The hearts were trimmed to remove the atria, and frozen in freezing compound (Tissue-Tek, Sakura Finetek USA Inc., Torrance CA USA) on dry ice and stored at -80°C . The hearts were sectioned to provide $10\text{ }\mu\text{m}$ thick sections at $200\text{ }\mu\text{m}$ intervals, which were viewed in fluorescence microscopy images.

For the histological measurements, the hearts were fixed with neutral buffered formalin applied via the aorta at a pressure of 15 mmHg ($20\text{ cm H}_2\text{O}$), which fixed the hearts in an end-diastolic configuration with dimensions approximating the dimensions observed by echocardiography (Whittaker and Patterson, 2000). Histological slides were processed at the Histology Core of the University of Michigan Dental School with Masson's trichrome staining to show fibrosis. The approximate percentage of the treated areas which showed scarring was assessed by image analysis of the blue (collagen) staining, excluding normal blue staining regions, relative to the red-stained healthy myocardial tissue.

Mason's trichrome stained sections were evaluated by measuring the fraction of fibrotic tissue in a 3 mm wide section within the treated region, where the width was set from estimated effective therapeutic beam width (Zhu et al. 2015b). The left ventricular wall thickness centered at the region of fibrosis was measured. For slightly oblique sections having an elliptical shape, for which the wall thickness was increased at the major axis ends of the ellipse, the measurements were corrected by normalizing the thickness measurement to the (untreated) minor axis thickness. In addition, immunohistochemistry was used to evaluate the progress of healing, which was expected to be incomplete at 28 d post treatment. Ongoing cardiac fibroblast activity adds numerous cells to the treated region, which prevents complete thinning of the wall until healing is complete. Fibroblasts involved in remodeling were identified by vimentin antibodies (Dako North America, Carpinteria, CA USA), which stain fibroblasts and endothelial cells (Travers et al. 2016).

Tests with the SS-16^{BN} model of HCM

Male SS-16^{BN} rats were purchased from the Medical College of Wisconsin. Rats were identified by ear tags and randomly assigned to treatment or sham groups. An acute study was planned as a baseline test (without methylprednisolone or losartan treatment) to assess HCM characteristics and the tolerance and effectiveness of the MCET treatment. Evans blue staining was observed in frozen sections at 1 d, and histological evaluation utilized heart samples collected at 2 d post treatment. The 12 rats were assigned as 2 sham and 4 treated for both the Evans blue test and the histological evaluation. The treatments involved 10 min of ultrasound exposure during microbubble infusion, while sham treatment involved ultrasound exposure for 10 min followed by microbubble infusion for 10 min without ultrasound. Data collected during treatment included heart rate, SpO_2 , and numbers of PCs in the ECG induced at the pulse triggers. The thickness of the left ventricular wall at the point of treatment was measured by echocardiography at the start of treatment, 1 d and 2 d after treatment.

The chronic study was planned to test the potential treatment efficacy for HCM therapy, seeking reduced myocardial wall thickness and function within the treatment volume at 28 d post treatment. Adjuvant treatment with methylprednisolone and losartan were included in

the protocol as describe above for all rats. The sham and treatment regimens were the same as for the previous studies with shams having 10 min ultrasound exposure without microbubble infusion, followed by 10 min of microbubble infusion. The treated group was set up to be larger than the sham group to allow for better assessment of the variable treatment effect. A total of 8 sham treatments and 14 therapy treatments were performed.

Results are reported as the means plus or minus one standard deviation, or plotted with standard error bars. Statistical analysis was performed (SigmaPlot for Windows V. 11.0, Systat Software Inc., San Jose CA, USA) to assess the statistical significance ($p < 0.05$) of measured differences. T-tests were performed for most comparisons. The Mann-Whitney rank sum test was performed for groups which failed the normality or equal-variance tests, which was typically found for the comparison of PC results. Paired tests were performed for data sets from the same rats, such a baseline ultrasound wall thickness compared to 1 d.

Results

Acute tests

Results of the acute study without methylprednisolone or losartan treatment are listed in Table 1. Unfortunately, three of the six rats given Evans blue died unexpectedly, apparently from an adverse reaction to the dye. MCET exposure resulted in PC generation for essentially all triggers and an average of about 30 ng/ml of troponin release. LV wall thickness was also significantly increased by 98% in treated animals at day 1 (2 d measurements were not significantly different from 1d). The Evans blue result was similar to previous work (e. g. Fig. 4 in Miller et al. 2014), indicating substantial myocyte killing in the target region. Histological observation revealed that the swollen area had large numbers of inflammatory cells in the regions of lethally injured cardiomyocytes. An example of the treated region after 2 d is shown Figure 1; this swollen region appears lighter than the surrounding tissue. Of note, the histological features other than hypertrophy of the SS-16^{BN} rats were similar to the Dahl SS rats and normal for rat hearts, with no myofibril disarray, incongruent with that seen in the human condition.

Chronic tests

The SS-16^{BN} rats (48–57 w old) in the chronic study had an average baseline LV wall thicknesses of 2.1 ± 0.15 mm, which was 31% ($p < 0.001$) and 17% ($p < 0.001$) larger than the preliminary (8–9 w) and acute (28–29 w) study rats, respectively, indicating substantial hypertrophy with age in this HCM model. Echocardiograms comparing sham and treatment images are shown in Fig. 2, with evident wall swelling at 1 d and wall thinning at 28 d within the treated myocardium. Measured parameters at treatment and 1 d are listed in Table 2, separated into suboptimal and optimal treatments (see below). The results for the echocardiographic wall thinning measurements are shown in Fig. 3. For shams, the wall thickness was essentially constant. For MCET treatment, the mean wall thickness was increased by 65% at day 1, relative to day 0, indicating a reduction ($p < 0.001$) in the 1 d thickening of about 35% by methylprednisolone adjuvant treatment, compared to the acute test without methylprednisolone.

During one treatment day with 4 rats, a reduced production of PCs was noted, and these were later found to have reduced MCET impact. This subset of the results for treated rats, indicated in Fig. 3, had suboptimal results of negligible wall thinning. When the wall thickness results were analyzed in terms of the PC production (Fig. 4), the suboptimal treatment outcomes were clearly separated from the optimal outcomes which had >90% PC production. Likewise, the troponin measurements (Fig. 4), which were proportional to wall thinning ($r^2=0.82$), show that the suboptimal treatment outcomes had troponin release <10 ng/ml. Therefore, the PC production and troponin measurements appear to be valuable predictors of optimal or suboptimal treatment results, which should be valuable for gauging treatment progress and adjustment of the treatment duration to achieve the desired overall reduction. The troponin test provides an indication of the expected wall thinning, which was variable between rats (Fig. 5).

In the observations of heart tissue after 28 d, some fibrosis was evident on surface of the heart in the target regions, and in histological sections as shown in Fig. 1. The echo wall thickness reduction of 16.2 % (n=11) was statistically significant ($p<0.01$), relative to the shams (n=8). Detailed results, separated into optimal or suboptimal cohorts, are listed in Table 3 for 28 d. The statistical analysis (given in the tables) clearly shows that the sub-optimal and optimal groups were significantly different. The echocardiogram LV wall measurements for optimal MCET animals showed myocardial reduction of 27% after 28 d relative to shams. For combined optimal and suboptimal groups, the histologically determined wall thickness was not significantly reduced. However, the histologically determined wall thinning in the optimal group of 16% was statistically significant. Suboptimal MCET animals failed to show a difference in myocardial thickness, suggesting inadequate treatment. ECG evaluation revealed the expected PCs at the time point of a treatment pulse burst and also included S-T segment elevation, multiple PCs, or bigeminy, which disappeared soon after cessation of treatment, as previously observed (Lu et al. 2016). No heart block was observed.

The endocardial displacement measurements shown in Fig. 5 also have a large significant decrease ($p<0.01$) of 29% at the treatment region, indicating true reduction in motion into the LV (as might be required for reduction of outflow track obstruction). The displacement decrease at 1d would likely minimize the potentially adverse consequences of the swelling. The overall heart function was evaluated by ejection fraction measurements, listed in Table 3. The ejection fraction was approximately the same for all groups at baseline, and was unchanged at 28 d for the shams and suboptimal treatments. However, for the optimal treatment there was a small reduction (10%, $p < 0.005$) in ejection fraction relative to shams, which was related to the displacement reduction.

The fractional strain in the heart wall at the treatment zone is plotted in Fig. 5. The strain was decreased by 34% ($p<0.005$) relative to shams. For successfully treated animals, the strain was decreased by 67% at 1 d (due in part to the swelling seen at 1 d), and remained decreased by 37% at 28 d ($p=0.001$). The mean fraction of fibrotic area in the treatment region, shown in Fig. 1, was 42% (Table 3). A closer examination of the treated region at 28 d is shown in Fig. 6, where an H&E stain reveals numerous fibroblasts still present in the healing tissue; confirmed by immunohistochemical localization with vimentin. The

numerous fibroblasts indicate that the healing process was not completed, with further wall thinning expected as the fibroblasts leave the area.

Discussion

Strategies for cavitation enabled therapy with ultrasound have been pursued extensively. Histotripsy applies very high amplitude pulses to initiate cavitation and homogenize tissue for non-invasive surgical applications (Khokhlova et al. 2015). Stabilized microbubbles used as ultrasound contrast agents can also produce localized biological effects adaptable for drug or gene delivery (Liang and McPherson 2009; Stride and Coussios, 2010; Bader et al. 2016). Ultrasound cavitation in heart tissue aided by contrast agent microbubbles for nucleation can produce scattered microlesions encompassing a few lethally injured cardiomyocytes. We have developed this micro-surgical phenomenon as a novel means (myocardial cavitation enabled treatment, MCET) to non-invasively reduce heart tissue in hypertrophic cardiomyopathy (HCM). Previous work with normal Dahl SS rats had demonstrated substantial tissue impact, with minimal impact on heart function after healing, but no clear tissue reduction (Lu et al. 2016). Reports of improved wall thinning after infarct (Roberts et al. 1985; de Carvalho Frimm et al 1997) and our preliminary testing suggested that MCET could be refined by adjuvant treatment with methylprednisolone and losartan to help reduce the initial swelling of the treated volume and possibly reduce fibrosis, see the Supplemental Material. In this study, myocardial cavitation enabled therapy (MCET) plus adjuvant treatment was evaluated in a genetic rat model of HCM.

The consomic Dahl SS rat with chromosome 16 from brown Norway rats (SS-16^{BN}) was used as the HCM model (few of which are available for rats) (Kriegel and Greene, 2008). The SS-16^{BN} rats proved to be difficult subjects with substantial problems related to the genetic disease in addition to the normal vicissitudes of *in vivo* ultrasound therapy research. Although the Evans blue dye was not well tolerated, the acute study showed that the treatment was reasonably well tolerated, and could be highly effective in these rats. After the acute study, the rats were housed to allow the myocardial hypertrophy to become significant. Although the rats developed hypertrophic hearts, the characteristic histological features of outflow obstruction and myofibril disarray was absent. During this time, 7 rats died, apparently of natural causes (and another excluded from the study due to illness). In addition, during the chronic study, 3 of 14 rats died after the MCET treatment, but none after sham treatment.

The chronic study was limited to 28 d to minimize the expected deaths in these old rats. Nevertheless, the chronic study was completed in 48–57 week old rats with 8 sham and 11 treated. As noted above, a subset of 4 rats, which were about 2 months older than the others, actually had less than expected PC generation and treatment response (suboptimal). Interestingly, the 4 suboptimal treatment rats were all in the same age group as the 7 rats lost during the delay between the acute and chronic studies. The microbubble suspension and exposure system were checked and found to be alright, which suggests that the age might have been the factor leading to the suboptimal results and the deaths. The other 7 rats, which had optimal treatment, demonstrated a significant and therapeutically useful magnitude of tissue reduction observable by echocardiography (27%) and histology (16 %) relative to

shams. Furthermore, the heart wall strain and endocardial displacement were reduced at 28 d by 37 % and 41 %, respectively, relative to shams. The results indicate a sufficient treatment efficacy for therapy, including a potential for the critical inhibition of outflow tract obstruction in symptomatic patients. The fibrosis areas observed at 28 d was substantial, but likely would be further reduced with the departure of fibroblasts and ultimate healing.

For comparison, in human HCM patients with surgical myectomy, the interventricular wall thickness was reduced by 18% from 19.4 to 15.9 mm (Brown et al. 2011). Alcohol induced septal thinning develops gradually after alcohol treatment and reductions from 23.7 to 18.0 mm (24%) have been reported after 6 months (Dabrowski et al. 2012). MCET may be more benign over the long term than either myectomy or alcohol ablation, since it preserves the cardiac architecture (vasculature, muscle fiber orientation layers) by targeted reduction rather (than gross deletion). This novel method may have the potential to supplant both currently accepted methods.

This study demonstrated MCET treatment response sufficient for advantageous therapy. Further research in rats is needed to understand the treatment variability, and to assess occurrence of adverse treatment outcomes (lack of reduction and unexpected deaths) seen in this study. Aside from a possible involvement of anesthesia, which suppressed the cardiovascular system (see Table 1), the procedure may have involved unanticipated side effects. For example, severe hemolysis with clinical side effects has been reported for ultrasound therapy in venous blood without anticoagulant, and this may be a risk with MCET (Devanagondi et al. 2015). Real time treatment assessment may allow better treatment management. The feedback provided by the PCs in the ECG together with plasma troponin measurements give predictive treatment feedback (in addition to the possible relief of symptoms in patients). However, cavitation activity and bioeffects can be monitored via the cavitation emissions, known as passive cavitation detection (Haworth et al. 2015; Crake et al. 2016). Adaption of a cavitation monitoring and mapping system for MCET in patients would allow real time treatment monitoring for desired distribution and overall efficacy.

We have demonstrated significant local myocardial reduction but still lack unequivocal evidence of therapeutic benefit. Rats are simply too small to investigate targeted reduction of outflow tract obstructions, even if a realistic rat model was available. The size also prevents evaluation of methods to treat larger volumes by beam scanning or to map the distribution of microlesions within a target volume. Human testing or a larger animal model of HCM similar to the human disease is needed for verification of safe and effective treatment outcomes. The testing of MCET treatment in humans seems premature without evidence of true therapeutic outcomes in a larger animal model. One excellent prospect for a larger animal model is feline breeds that develop fatal HCM. The genetic characteristics are similar in cats and humans and the feline disease closely models the human (Kittleson et al. 1999; Kittleson et al. 2015; Fox et al. 2014). The feline model appears to be a suitable next step in developing MCET for myocardial reduction therapy, which can be conducted in veterinary hospitals for sick cats presenting with life threatening HCM.

Conclusions

A new non-invasive method for myocardial tissue reduction would be a valuable addition to HCM treatment options. Ultrasound myocardial cavitation enabled treatment is an image-guided method capable of graded and diffuse tissue reduction with potentially minimal adverse side effects, relative to alcohol ablation or invasive surgery. This study showed that optimal MCET could reduce wall thickness by about 27% and endocardial border displacement by about 37%. After further development and clinical translation of the treatment system, MCET micro-surgical therapy may provide an alternative, non-invasive treatment option for patients with HCM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Photographs of a treated SS-16^{BN} rat heart from the acute and chronic studies. The light area in the heart (A) is the treated region with inflammation after 2 d. An H&E stained histological section (B) shows the wide region of inflammatory swelling (arrows). The blue color of the swollen region is due to numerous inflammatory cells with blue-stained nuclei. An optimally treated rat heart from the chronic study after 28 d. The light area in the heart (C) is the treated region with fibrous tissue appearing white. A Masson's trichrome stained histological section (D) from the center of the treated region with fibrotic (scar) tissue at the treatment target (arrow) stained blue with Masson's trichrome stain. The targeted region of the left ventricular wall is noticeably reduced in thickness. The scale bars are 5 mm.

Figure 2.

Echocardiographic images of a sham-treated rat (top row) and a treated rat (bottom row) from the chronic study (8 sham and 14 treated rats). The time points were baseline (left column), 1 d (middle column) and 28 d (right column). The arrows in the left ventricle of the treated heart approximately indicate the treated region in the anterior myocardium, which had inflammatory swelling in a region larger than the beam path at 1 d and the narrower thickness reduction at 28 d.

Figure 3.

Results for echocardiographic LV wall thickness from the chronic study. The echocardiographic results for the treated rats (blue symbols) $n=14$ was significantly increased above shams (black symbols) $n=8$ at day 1 and significantly reduced at day 28 (**, $p<0.001$). Standard error bars (green) include all data points. A suboptimal subset of the treated rats (red symbols) had no wall thinning.

Figure 4.

Premature complex (PC) yield for the ultrasound pulse triggers (top) and the plasma troponin release (bottom) plotted as a function of the echocardiographic wall thinning. The PC data ($n=19$) has a sigmoidal trend ($r^2=0.83$). The linear regression for the troponin results ($r^2=0.82$) ($n=19$) shows proportionality to wall thinning. The low results for the suboptimal treatment (red symbols) recognized in Fig. 3 are clearly differentiated from the successful treatments (blue symbols) and shams (green symbols). Note that some shams have negative thinning (i. e., thickening) due to continued hypertrophy.

Figure 5.

Analysis of echocardiography for the endocardial wall displacement (top) and the heart fractional strain in the treated region (bottom) plotted as in Fig. 3 with standard error bars in green. The treated wall displacement was significantly reduced ($n=11$) after 1d when the tissue was somewhat swollen, and remained significantly reduced after 28d (*, $p<0.05$). Sham treated results were unchanged ($n=8$). A similar trend was even more distinct for wall strain (**, $p<0.005$). The sub optimal treatments (red symbols) had somewhat less impact relative to shams (black symbols) on these measures than did the optimal-treatments (blue symbols).

Figure 6.

Histological images from a sham-treated rat heart (top row) and from an optimally treated heart (also shown in Fig. 1B) (Bottom row) from the chronic study (8 sham and 14 treated rats). Staining methods were hematoxylin and eosin (left column), Masson's trichrome (middle column) and immunohistochemical staining with vimentin antibodies (right column). With hematoxylin and eosin, or Masson's trichrome staining, many more cell nuclei (blue dots) are still evident in the fibrotic regions (blue with Masson's trichrome). These cells are identified as numerous additional small fibroblasts and some endothelial cells in capillaries by the vimentin staining. The scale bar of 200 μm is the same for all images.

Results of the acute test of MCET in the SS-16^{BN} rats during treatment. Echocardiographic measurement of the left ventricular wall thickness at the point of treatment was performed at treatment (0 d) and 1 d after treatment.

Table 1

Test	n	Weight gm	HR BPM	SpO ₂ %	Triggers	PCs	Troponin ng/ml	Echo wall (mm)	
								0 d	1 d
Sham	3	443 ± 16	252 ± 10	78 ± 7	273 ± 15	0	0	1.80 ± 0.07	1.91 ± 0.14
Treated	6	460 ± 25	249 ± 18	79 ± 5	254 ± 16	253 ± 17	29 ± 11	1.82 ± 0.07	3.61 ± 0.33
p		nsd	nsd	nsd	nsd	<0.001*	0.003	nsd	<0.001

No significant difference: nsd

* p value from Mann-Whitney rank sum test.

Table 2

Results for the treatment of the chronic test of MCET in the SS-16^{BN} rats, separated into optimal (OP) and suboptimal (SO) groups. Echocardiographic measurement of the left ventricular wall thickness at the point of treatment was performed at treatment (0 d) and 1 d after treatment. The OP and SO groups had significantly different PC and troponin results.

Test	n	Weight d0 gm	Weight d28 gm	Triggers	PCs	Troponin ng/ml	Echo wall (mm)	
							0 d	1 d
Sham	8	448 ± 21	445 ± 24	279 ± 16	0.75 ± 1.0	0.0	2.07 ± 0.13	2.07 ± 0.11
Treat SO	4	467 ± 11	449 ± 15	280 ± 11	192 ± 12	5.4 ± 2.0	2.16 ± 0.14	3.35 ± 0.24
P		nsd	nsd	nsd	0.004 *	0.004 *	nsd	0.004 *
Treat OP	8	439 ± 29	429 ± 27	289 ± 12	284 ± 17	17.6 ± 6.8	2.07 ± 0.23	3.53 ± 0.23
P		nsd	nsd	nsd	<0.001 *	<0.001 *	nsd	<0.001
SO vs OP		nsd	nsd	nsd	<0.001	0.005	nsd	nsd

No significant difference: nsd.

* p values from the Mann-Whitney rank sum test. P value between the SO and OP treated groups; SO vs OP.

Table 3

Results for the 28 day follow-up after treatment in the chronic test of MCET in the SS-16^{BN} rats, separated into optimal (OP) and suboptimal (SO) treatment groups which had significantly different results.

Test	n	Ejection Fraction					Displace. mm	Strain fraction	Fibrotic Area %
		baseline	28 d	Echo wall mm	Histo wall mm				
Sham	8	78 ± 3	76 ± 4	2.19 ± 0.20	2.74 ± 0.32		0.86 ± 0.21	0.44 ± 0.10	9 ± 4
Treat SO	4	80 ± 4	77 ± 4	2.09 ± 0.05	3.08 ± 0.28		0.73 ± 0.10	0.35 ± 0.07	33 ± 5
P		nsd	nsd	nsd	nsd		nsd	nsd	<0.001
Treat OP	7	76 ± 3	68 [‡] ± 4	1.61 ± 0.22	2.29 ± 0.33		0.54 ± 0.14	0.26 ± 0.06	42 ± 10
P		nsd	<0.005	<0.001	0.017		0.009 [*]	0.001	<0.001
SO vs OP		nsd	<0.01	0.002	0.003		0.003	0.05	nsd

No significant difference: nsd.

[‡] significantly different from baseline.

^{*} p value from the Mann Whitney rank sum test. P value between the SO and OP treated groups: SO vs OP.