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The Use of Theranostic Strategies in Myocardial Cavitation-Enabled Therapy

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Abstract

The accumulation of microlesions induced by ultrasound interaction with contrast microbubbles in the myocardium potentially represents a new method of tissue reduction therapy. Anesthetized rats were treated in a heated water bath with 1.5 MHz focused ultrasound pulses triggered once every 4 heartbeats from the electrocardiogram (ECG) during infusion of microbubble contrast agent. Treatment was guided by an 8 MHz B-mode imaging transducer, which also was used to provide estimates of the left ventricular echogenicity (LVE) as a possible predictor of efficacy during treatment. Strategies to reduce prospective clinical treatment durations were tested, including pulse modulation to simulate a theranostic scanning strategy, and an increased agent infusion rate over shorter durations. Sources of variability, including ultrasound path variation and venous catheter placement, also were investigated. ECG premature complexes (PCs) were monitored and Evans-blue stained cardiomyocyte scores (SCSs) were obtained from frozen sections. The LVE reflected variations in the infused microbubble concentration, but failed to predict efficacy. Comparing suspensions of varied microbubble sizes revealed that LVE was dominated by larger bubbles, while efficacy appeared to be dependent on smaller sizes. Simulated scanning was as effective as the normal fixed-beam treatment, and high agent infusion allowed reduced treatment duration. The success of these theranostic strategies may increase the prospects for realistic clinical translation of myocardial cavitation enabled therapy.

Keywords

Myocardial Contrast Echocardiography; Arrhythmia; Cardiac Myocyte Necrosis; Hypertrophic Cardiomyopathy

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Introduction

The exploration of the ultrasonic activation of microbubbles in the circulation for therapeutic purposes continues to grow (Unger et al. 2014; Aryal et al. 2014; Ibsen et al. 2013a; Sun et al. 2014). Ultrasound microbubble-activation therapies have attractive features, including the ability to guide the treatment using ultrasound imaging, and real time feedback from the microbubble emissions (Cochran et al. 2011; Kooiman et al. 2014; Vignon et al. 2013). Prospective treatments range from transient opening of capillaries, such as the blood brain barrier (Burgess and Hynynen, 2014), to complex methods for drug or gene delivery using specially prepared gaseous delivery vehicles that can be targeted to the desired region for treatment (Ma et al. 2013). These methods have been studied for treatments such as gene therapy, cancer treatment, and thrombolysis (Laing and McPherson 2009; Ibsen et al. 2013b; Unger et al. 2014). However, these possibilities have significant barriers to clinical translation of laboratory methods, for example in drug delivery (Lanza et al. 2014), and further intensive research is needed on these and other novel treatment ideas to improve prospects for translation to the clinic.

Recently we have proposed the possible use of contrast agent microbubbles for producing scattered sites of ultrasonic cavitation nucleation and microlesion production in the heart, which may provide a relatively safe means of tissue reduction therapy (Miller et al. 2014a). This myocardial cavitation-enabled therapy (MCET) should allow a graded treatment with ultrasound image guidance and healing with minimal scar formation. This treatment method could be beneficial for hypertrophic cardiomyopathy (HCM) and other ventricular hypertrophies. HCM is the most common genetic cardiovascular disease, occurring in 1 in 500 people (Maron et al. 1995). The hypertrophy can lead to asymmetric enlargement of the intraventricular septum with obstruction of the left ventricular outflow tract. Young athletes with HCM may be completely asymptomatic until vigorous exercise induces cardiac symptoms including even sudden death (Ommen and Nishimura, 2004). The optimum method for diagnosis, including screening of athletes, is echocardiography that can image and characterize the hypertrophy and potential obstruction. As many as one-third of patients become candidates for myocardial reduction (Marian, 2009), which is typically accomplished by septal myectomy (Maron et al. 2003). Other technologies have been studied as possible alternatives to heart surgery for HCM. Thermal ablation with high intensity focused ultrasound (Otsuka et al. 2007), radio-frequency catheter ablation (Lawrenz et al. 2011) and transcatheter alcohol ablation (Leonardi et al. 2010) have been explored with limited success and often with added risk of arrhythmia (Marian 2009, Nishimura and Ommen, 2010). A less-invasive, safer alternative to surgery or total regional ablation remains elusive, but would represent a welcome treatment option.

In the MCET method, ultrasound contrast agent microbubbles are continuously infused intravenously, while intermittent contrast destruction and refill cycles are completed using focused therapy transducers, gradually building up the desired level of microlesion density. The adjustable treatment process allows for minimization of the physiological impact on cardiac function during treatment. The end of systole was identified as an optimal trigger time point that produced identifiable electrocardiogram (ECG) premature complexes (PCs) and substantial cardiomyocyte injury, but minimal cardiac functional disruption (Miller et al.

2014b). A problem for optimization of the treatment due to uncertainty in the visual scoring of microlesion accumulation after therapy-level treatment seems to be amenable to quantitative image analysis. Three dimensional analysis of stacked tissue sections showing microlesions provides an effective means to characterize the overall impact or “macrolesion” created in the treated volume (Zhu et al. 2015).

This study aims at making the MCET method more amenable to clinical translation by enhancing the technique in three ways: reducing the duration of the HIFU exposure, adding real time control of lesion formation, and providing accurate measures of microbubble dose in the myocardium.

Materials and Methods

Approach

To achieve the first objective of reducing HIFU exposure duration, a Gaussian pulse modulation envelope was tested to gauge the potential efficacy of treatment with a rapidly scanned therapy beam. Previously, 5 min exposures were used to treat a single focal spot, which would entail prohibitively long duration treatments even for small tissue volumes. The Gaussian modulation simulated the pulse-sequence modulation seen with a scanned beam, as in diagnostic imaging (Miller et al. 2007). The use of scanned beams for therapy treatments would be fast and accurate for covering a target volume, combining imaging and treatment. The strategy has been called “theranostic” ultrasound (Martin and Dayton, 2013), and represents a promising means of creating cavitation enabled therapies suitable for clinical application. The desired treatment volume for hypertrophic cardiomyopathy already can be identified and mapped for surgery using three dimensional ultrasound (Sadat et al. 2013). The prospective theranostic method for MCET would simply add a high-power scan region set in the image to include the volume targeted for tissue reduction.

Another requisite goal will be to control treatment progress in real time and to predict the numbers and distributions of microlesions within hypertrophied regions of the myocardium. In small animals, the real time control of MCET involves observation of at least three possible control predictive indicators for guiding adjustment of exposure factors. One is the provision of a consistent ultrasound path for the therapeutic beam to the myocardium, which can be accomplished by image guidance. Our system employs an 8 MHz sector scanner to thread a 1.5 MHz fixed-beam through the “window” between two ribs, and between the sternum and left lung lobe (Miller et al. 2014a). However, this is approximate, and some means to monitor the transmission losses to the heart would be of value. Another factor is the occurrence of the PCs in the ECG signal, which are strongly correlated with cardiomyocyte injury (Miller et al. 2011). Previously, this easily monitored indicator was found to be useful for assessing whether or not effective conditions were active, but the relationship to the actual total effect was problematic for highly efficacious conditions (Miller et al. 2014b).

A third factor is the microbubble dose actually delivered to the myocardium. In previous research on capillary injury in kidney, the delivered dose was found to be variable from animal to animal, and also during the treatment due to microbubble destruction (Miller et al.

2010). For this study, we hypothesized that the microbubble-laden blood in the left ventricle provides a real time observable indicator of circulating microbubble dose, including destruction and refill. This hypothesis was examined by analyzing images of the left ventricle to measure the echogenicity of image regions set entirely within left ventricular blood. In addition, the influence of modified microbubble suspensions with enhanced small or large bubble populations was assessed by comparing left ventricular echogenicity (LVE) and treatment efficacy. The results provided several valuable insights, which help to refine the MCET method.

Animal preparation

All *in vivo* animal procedures were conducted with the approval and guidance of the University Committee on Use and Care of Animals of the University of Michigan. Forty-three Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were used in the study, with 2 excluded from data collection due to death prior to sample acquisition. The 41 remaining rats weighing an average of 352 ± 30 g were anesthetized by intraperitoneal (IP) injection of a mixture of ketamine (90 mg kg^{-1} , Ketaset, MWIVeterinary Supply, Boise, ID) and xylazine (9 ml kg^{-1} , MWIVeterinary Supply). The left thorax was shaved and depilated for ultrasound transmission. A 24 gauge cannula (BD Angiocath, Becton Dickinson Infusion Therapy Systems, Sandy, UT, USA) was inserted into a tail vein or jugular vein for IV injections of contrast agent. Evans blue dye (E1129, Sigma-Aldrich Co, St. Louis, MO, USA) in saline (20 mg/ml) was injected IV via tail vein at a dose of 100 mg/kg as a vital stain for cardiomyocytes (Miller et al. 2005). The rats were mounted on a positioning board and ECG needle electrodes (Reusable ECG Electrodes, C3301572, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) were placed in the forelegs and left hind leg. The rat and board were then mounted for ultrasound exposures in a 37°C degassed and deionized water bath. The ECG signal was acquired (Electrocardiogram (ECG) Amplifier Module (ECGA), 73-0149, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany), digitized, and analyzed with the aid of software (PowerLab-4 receiver and Chart Pro 5, v. 5.5.5, ADInstruments Inc., Colorado Springs, CO, USA), as described in previous work (Miller et al. 2014a). The average heart rate was 250 ± 39 bpm. The ECG provided data on the response to the ultrasonic therapy in terms of premature complexes (PCs), which coincided with the trigger of a treatment pulse burst and are strongly correlated with the injury of cardiomyocytes (Miller et al. 2011).

Ultrasound

Ultrasound exposure for treatment was provided by a laboratory system with guidance by diagnostic ultrasound imaging, as described previously (Miller et al. 2014a). 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), a power amplifier (A-500, Electronic Navigation Industries, Rochester NY, USA) and a 1.5 MHz single element treatment transducer (Panametrics A3464, Olympus, Waltham, MA, USA). The damped 1.9 cm diameter treatment transducer had a 3.8 cm focal length, with a -6 dB beam diameter of 3.5 mm. The treatment was targeted with the aid of diagnostic ultrasound imaging (GE Vivid 7 with S-10 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 5-cycle pulses at a 4 kHz pulse repetition frequency. The peak rarefactional pressure amplitudes of the pulses were measured using a calibrated hydrophone (model 805, Sonora Medical Systems Inc., Longmont CO USA), and a 4 MPa peak rarefactional pressure amplitude was obtained. The amplitude modulation was set to give zero exposure unless a modulation-envelope signal was triggered. The envelope signal was either a 2 ms square, which gave a burst of 8 pulses as used previously, or a Gaussian function, which gave 2 ms duration of pulse amplitudes greater than one-half the peak (-6dB). The pulse groups are shown in Fig. 1. The Gaussian modulation simulates the pulse amplitudes expected from a rapidly scanned focal zone, as for B mode sector scanning at about 56 fps, which would be desirable in a theranostic strategy for clinical implementation. The modulated pulse bursts were triggered from the ECG signal at approximately end of systole each 4 heart cycles, as described previously (Miller et al. 2014a).

Left ventricular echogenicity measurement

Miller et al. (2014a) found that there was considerable variability in the outcomes of similar treatments, which might be detrimental to well controlled MCET treatment. Part of this variability was thought to result from variations in the ongoing circulating concentration, or dose, of the ultrasound contrast agent. A previous study of contrast agent dose in kidneys showed that diagnostic ultrasound exposure with microbubble destruction greatly reduced the circulating concentration (Miller et al. 2010). Therefore this phenomenon must be an important factor for MCET treatment, particularly in small animals.

For this present work, the imaging probe was used to briefly acquire images of the left ventricle using a reduced power setting of -12 dB (Mechanical Index 0.2). Images of the left ventricle echogenicity (LVE) are shown in Fig. 2a without contrast enhancement and in Fig. 2b with contrast enhancement. Note the substantial enhancement of echogenicity within the ventricle with added contrast agent, which provided an indication of the microbubble concentration in the ventricle during treatment, and thus the dose reaching the myocardium. The Quantitative Analysis package on the Vivid 7 machine was used to generate time-intensity curves, in terms of linear acoustical units (AU) of echogenicity, from a region of interest (ROI) within the left ventricular image. The acoustical units were derived internally by the machine by decompressing the logarithmic (i. e. dB) image brightness data and were linearly proportional to acoustic intensity, i.e. W/cm^2 . These units will be reported in here as mAU since the measured values reside close to 0.001 AU. To ensure that the region was entirely within the blood, a small oval region about 2 mm lateral by 3 mm axial was set, which avoided the very bright intrusion of lung or bone echoes. This produced somewhat noisy time-intensity curves, as shown in Fig. 3. By manually searching the AU values during the heart cycle, an estimate was made of the mean LVE at approximately end diastole. For Fig. 3, this LVE result was 50 mAU. During a treatment, the LVE was measured for 2–3 heart-cycles before starting the agent infusion, 30 s after starting infusion, during treatment

about 30 s after starting, and 30 s after the end of treatment but with the infusion still running. The latter time point was compared with the first time point before treatment to assess any variation of the agent dose during the procedure.

Ultrasound contrast agent

The ultrasound contrast agent Definity® (Lantheus Medical Imaging, Inc., N. Billerica, MA) served as the source of in vivo cavitation nuclei for MCET with a vial used for one week (two treatment days, typically with three rats each). This was diluted in sterile saline in a 3 ml syringe, and infused via tail vein with a syringe pump. The infusion rate of Definity was 5 $\mu\text{l/kg/min}$ or 12.5 $\mu\text{l/kg/min}$ for therapy exposures, or variable for image calibration (see below). The 5 $\mu\text{l/kg/min}$ rate was employed previously (Miller et al. 2014a) and represents a maximal recommended dose for diagnostic application. The 12.5 $\mu\text{l/kg/min}$ rate was tested to explore the possible use of higher doses in therapeutic applications, which might allow for reduced treatment durations.

Initial studies indicated that the LVE for Definity infusion did not appear to track the myocardial treatment efficacy as well as had been expected. A hypothesis was formulated to help explain these initial results and proposed that the larger bubbles were dominant for the LVE but smaller microbubbles supplied most of the cavitation nucleation. To test this idea, contrast agent suspensions were fractionated to enhance the population of large or small microbubbles. Relatively large amounts of contrast agent were needed to perform the fractionations, and a substitute for Definity was created. 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 lipids were dissolved in a diluent consisting of 80% (v/v) phosphate buffered saline, 10% (v/v) propylene glycol, and 10% (v/v) glycerol at concentrations of 0.045 mg/ml, 0.4 mg/mL, and 0.3 mg/mL for DPPA, DPPC, and DPPE-PEG500, respectively. The mixture of lipids and diluent was heated to 70° C (i.e. above the transition temperature of DPPA) to solubilize the lipids. The resulting lipid blend underwent sterile filtration using a 0.2 μm filter and then aliquoted into used Definity vials that were cleaned and autoclaved. 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. The vials were shaken exactly as performed for Definity to produce the suspensions of stabilized microbubbles. The substitute contrast agent (SCA) contained 3.8×10^9 microbubbles/ml of $1.8 \pm 1.2 \mu\text{m}$ diameter, similar to Definity (1.1–3.3 μm diameter range up to 1.2×10^{10} microbubbles/ml).

For the production of different size ranges, a simple centrifugation method was used (Feshitan et al. 2009). Briefly, 24 ml activated SCA was divided into three 10 ml syringe tubes and centrifuged for 1 min at 50 relative centrifugal force (RCF) and the large bubbles at the surface were discarded. The infranatant was centrifuged at 100 RCF for 1 min and the upper bubble layer was collected and resuspended in phosphate buffered saline (PBS). This process was repeated four times to reduce the small microbubble population. The final result was resuspended in 1.0 ml PBS as the large microbubble suspension. The infranatants from

this process were collected, centrifuged at 200 RCF for 1 min and the upper bubble layer was discarded. The remainder was centrifuged at 300 RCF for 10 min, and the upper layer was resuspended in 1.5 ml PBS as the small bubble suspension. The size distributions of the suspensions were measured with a Coulter counter (Multisizer 4, Beckman-Coulter Inc. Indianapolis IN USA) and are given in the Results.

Cardiomyocyte scoring

Lethal injury of cardiomyocytes was scored on the basis of Evans blue staining, as described previously (Miller et al. 2005; Miller et al. 2014a). Briefly, the hearts were removed the day after exposure and cleared of most blood with heparin-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 up to forty 10 μm thick sections at 200 μm intervals. Each slide was scored blindly using fluorescence microscopy and a final stained cardiomyocyte score (SCS) was calculated as the sum of the scores for the individual slides. For these therapeutic exposures, there were too many close packed stained cells to count individually; hence, the SCS was a qualitative measure of overall injury.

Experimental Plan

For exposure, the image was used to target the heart, and then the exposure transducer was moved into place. After aiming, the ECG was recorded for 1 min pre-exposure, 5 min during exposure with infusion of diluted contrast agent, and 1 min post-infusion with continued exposure. In addition, the ECG was recorded for 1 min before heart removal the next day. The imaging system was operated as described above to obtain the representative AU values for each treatment. After treatment, the electrodes and catheter were removed, and the rat was dried and monitored in a warmed cage until fully awake.

The study was conducted with groups of rats given letter designations and each treated with a specific set of conditions. Groups A-E were planned specifically to test the relation of LVE and stained cell scores, as listed in Table 1, for changes in treatment at 4 MPa. Comparison of groups A and B tested the influence of catheter placement, with the jugular vein giving central, larger vein access. Groups B and C were compared to test the effect of aiming near ribs. Groups B and D compared treatment using the square or Gaussian pulse train modulations. The use of a higher contrast agent dose rates was tested by comparing groups D and E. Finally, Group F was a sham and calibration control group, in which each rat received sham therapy exposure without contrast agent, followed by a graded series of infusion concentrations for 2 min each, all with LVE measurements. A low range of infusions utilized 100:1 dilutions to achieve 2.5, 5, or 7.5 $\mu\text{l/kg/min}$ with different pump rates, and a higher range utilized 40:1 dilutions to achieve 12.5 or 20 $\mu\text{l/kg/min}$.

Finally, a test of contrast agent suspensions with enhanced small or large microbubbles was conducted to gauge the influence of microbubble size on LVE and the SCS. Three groups of 4 rats were treated as for Group D described above, except that tail vein infusion was used. The groups received the normal SCA, SCA with small microbubbles enhanced or SCA with large microbubbles enhanced. Two preparations of the suspensions were used for 6 rats

each. During treatment, the LVE was measured and PCs counted. The SCSs were obtained from heart samples taken one day post exposure. The size-modified SCA suspensions appeared to be somewhat less stable than the normal SCA, but performed well enough to provide definitive information on the relative differences in LVE and SCS due to microbubble size.

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). Student's *t*-tests were used to compare means of the measured parameters, with statistical significance assumed at $P < 0.05$.

Results

The echogenicity results from group F are plotted in Fig. 4. The LVE was linearly related to the dose rate with a coefficient of determination $r^2 = 0.77$. This finding supports the hypothesis that the AU measurement provides a predictive proxy measure of the circulating dose of contrast agent. For groups A-D, the LVE before infusion averaged 0.7 ± 0.2 mAU, which was much less than the result after infusion 31 ± 13 mAU. These results had a substantial variability with standard deviations of about 30–40% of the means. The final measurements taken during infusion 30 s after the cessation of exposure averaged 25 ± 19 mAU, which was not significantly different from the measurement just before starting exposure. This indicates that the infusion dose rate was stable throughout each test (e. g. not declining due to instability of the microbubble suspension or other factors).

The echogenicity of the intervening skin and intercostal space (and possibly nearby bone or lung) might represent an indicator for attenuation, which could provide an adjustment factor to reduce variability. This was measured for groups A-D for an ROI directly above the left ventricle. The results for the intervening tissue are compared with the initial AU for infusion just prior to exposure in Fig. 5. There was a trend suggesting that low echogenicity of the intervening tissue corresponded with higher LVE; however, even when the two outliers (circled) were omitted, the coefficient of determination was low at $r^2 = 0.47$. The echogenicity of the intervening tissue was not used as an adjustment for the LVE due to this low correlation, and because its relationship to the attenuation for the therapy beam, which entered from a somewhat different angle, was unknown. However, this trend indicates that variation in intervening tissue scattering and attenuation might account for some of the variability in treatment results.

When the therapy exposure was begun, the LVE significantly declined. The time intensity curve for a contrast destruction and refill cycle from a group D rat is shown in Fig. 6. For the first heartbeat after the triggered pulse burst, the LVE was reduced to nearly the pre-infusion value. For the treatment imaging in Fig. 6, the pre-infusion and infusion AU values were 0.4 mAU (Fig. 2a) and 50 mAU (Figs 2b and 3). In Fig. 6, the lowest post destruction AU value at end diastole was 1 mAU, which recovered to 12 mAU after three beats (the missing beat was the premature complex induced by the pulse burst). For groups A–D ($n = 19$), the mean post-destruction value was 2.1 ± 2.5 mAU ($P < 0.001$ vs the pre-exposure value), and the mean

refill (pre-destruction) value was 7.7 ± 6.0 mAU ($P < 0.001$ vs the pre-exposure value and the destruction value).

The results for groups A–E are presented in Fig. 7. Two groups were compared for the pre-exposure AU, the refill AU just prior to the next destruction, the ratio of pre-destruction to pre-exposure AU, the percentage of pulse burst triggers resulting in PCs in the ECG, and the stained cell scores. For groups A and B, no significant differences were found, which indicates that the choice of vein for infusions was not a factor in the results. A comparison between groups B and C also had no significant differences, showing that small variations in aim were not important. The comparison between square and Gaussian pulse modulation also did not produce a significant difference in results. This indicates that the scan mode for theranostic treatment should perform well, with little loss of efficacy relative to the fixed multi-pulse treatment. Group E, which had increased contrast agent infusion rate but a shorter treatment duration of 2 min to equalize the total agent dose, had an increased pre-treatment AU ($P = 0.073$) relative to group D, but no significant differences. This result suggests that the higher agent infusion offers a means to shorten treatment times, at least in small animals.

The measurement of the LVE was intended to help predict the variations in the SCS results for nominally similar treatments. Two measures of the circulating treatment dose of contrast agent were the initial AU achieved by the infusion just prior to starting treatment, and the AU observed during treatment, after refill just before a pulse burst was triggered. The SCSs are plotted in Fig. 9 against the initial AU results. The linear regression line shows little dependence on the LVE, with a possible indication of a negative correlation ($r^2 = 0.13$). The SCSs are plotted in Fig. 10 against the refill AU results. The linear regression line shows no dependence on the LVE ($r^2 = 0.0023$). These findings clearly show that the treatment efficacy is not related to the LVE. A dependence coefficient could be calculated by forcing the fitted lines to pass through the origin, but the result would be of no value. For example, a procedure designed to adjust the initial AU or refill AU to a constant value in an effort to reduce the variability of the SCS would be futile using such an artificial factor.

This unexpectedly poor performance of the LVE for predicting efficacy led to the hypothesis that the larger bubbles were dominant for the LVE but smaller microbubbles supplied most of the cavitation nucleation. This hypothesis was tested using the comparison of normal SCA, and SCA suspensions with enhanced small or large microbubble populations. The enhancement process lead to reasonably distinct size ranges as shown in Fig. 11. A separation point of $2.5 \mu\text{m}$ diameter was chosen to categorize the suspensions with the percentages of large and small microbubbles listed in Fig. 10. Bubbles larger than $6 \mu\text{m}$ were excluded, since these would be filtered by the pulmonary circulation and not appear in the LVE. The LVE produced by the normal SCA was comparable to the LVE produced by Definity, see Fig. 3. The LVE of the large-size microbubble suspension was more than 100 times that of the small size suspension ($P < 0.007$), which was only about twice the pre-infusion background. The large size fraction clearly dominates the LVE and provides the strong LVE of the normal suspension. The percentage of PCs induced by the triggered pulse bursts were, in contrast, not significantly different for the three suspensions. Likewise, the SCSs were not significantly different for the large and small size suspensions due to the high

variability of the results; however, the more stable normal suspension result was somewhat larger than the small ($P<0.02$) and large ($P=0.07$) size suspensions.

Discussion

In this study, several considerations for a theranostic strategy were investigated for MCET (Table 1 and Fig. 7). The normal use of the rat tail vein was thought to be different from larger, more central veins. However, a comparison between tail and jugular vein catheters for infusion did not show a significant difference in results. The simple rat preparation using the tail vein infusion appears to approximate infusion via larger veins, as would be used in humans.

The use of a scanned exposure beam for MCET is essential for reducing the treatments to a reasonable duration for clinical application. This possibility was tested relative to the fixed beam approach by using a Gaussian modulation envelope for the pulse burst (see Fig. 1), which simulates exposure by a scanned beam. This condition performed as well as the previous square envelope modulation. Using a scanned beam would accelerate substantially the progress of treatment relative to treatment of single points (e. g. typically used for HIFU treatment). In addition, the ability to use higher (than typical diagnostic) microbubble infusion rates for reducing treatment periods was confirmed by comparing 5 min with 5 $\mu\text{l/kg/min}$ infusion to 2 min with 12.5 $\mu\text{l/kg/min}$. The theranostic strategy of image guided treatment by scanning a tissue volume therefore appears to be a realistic possibility.

A potential problem for the treatment, at least in rats, is the substantial variation in treatment results for seemingly identical conditions. One source of variation is the variation in attenuation and scattering by the intervening tissue. When the echogenicity of the tissue (skin, intercostal space) between the chest surface and the heart was measured, substantial variation was found (Fig. 6). The variation was modestly correlated with a decreasing readout of LVE, which suggests that the ultrasonic properties of the intervening tissue is important.

Another source of variation in treatment outcome was thought to be due to variation in the contrast agent dose actually reaching the circulation, which was estimated by scattering measurements for ROIs within the LV (Figs. 2 and 3). This pretreatment measure of LVE, expressed in acoustical units, was proportional to the infusion rate in sham-treatment tests (Fig. 4). The LVE was reduced substantially by the therapy, with a typical agent destruction and refill pattern (Fig. 5). This identified another important agent dose parameter, which was the LVE at refill, immediately before the pulse-burst trigger. The LVE results showed relatively large variation, with standard deviations often 30 percent of the means (Fig. 7), not unlike the substantial variation in the SCS results (Fig. 7). However, comparison of the LVE results to the pre-treatment and refill echogenicities to SCS results showed that the variation in LVE was not predictive of treatment outcome (Figs. 9 and 10). Therefore, the adjustment of the contrast AU readout of the LVE either before or during treatment cannot be used to reduce the variation in treatment results.

This finding was initially puzzling, because the cavitation microlesions produced by cavitation nucleation from the microbubbles should be related to their infusion. However, an important factor is the strong dependence of ultrasound scattering on microbubble size to the sixth power (Forsberg and Shi, 2001), such that large bubbles would generate most of the LVE. We hypothesize that the outcome truly is related to the infusion of the optimum cavitation nuclei, but that the measured echogenicity does not indicate the concentration of these nuclei. The results of the experiment using microbubble suspensions with enhanced small or large microbubble populations supported this hypothesis (Figs. 8 and 11). Since the normal suspension is dominated by the small size range microbubbles, most of the efficacy for normal suspensions must arise from the small microbubbles, which were nearly undetectable by the LVE measurements. The large microbubble fraction of the normal suspension, which must generate most of the LVE, therefore has only a small role in treatment efficacy. Variation in the relative population of easily destroyed large bubbles caused by mixing in a syringe or the vial, flotation, catheter constrictions, animal dependent removal in the lungs and possibly other perturbations would vary LVE, giving the very weak relationship to efficacy as shown in Figs. 9 and 10.

This finding of a problem with microbubble-dose monitoring may actually provide a solution for MCET. The use of contrast agent media that was modified initially to reduce the numbers of larger microbubbles would greatly reduce the microbubble scattering and consequent image shadowing. Therefore, higher micronuclei dosages could be employed to reduce treatment duration without image degradation. This feature of MCET may prove to be a key refinement needed for clinical success in humans, for whom scattering, attenuation and shadowing pose a much larger problem than for rats.

A better approach than the LVE for real time control of MCET impact may be passive cavitation monitoring. This has been demonstrated in vitro and in tissues (Salgaonkar et al. 2009; Haworth et al. 2012; Jensen et al. 2012). Imaging and mapping of cavitation nucleation sites can utilize harmonic, sub- and ultraharmonics and broadband emission from transient cavitation. The use of imaging probes has been demonstrated in heart, liver and brain (Vignon et al. 2013), and transcranial three-dimensional imaging has been shown to be feasible for monitoring blood brain barrier disruption (O'Reilly et al. 2014). Spatial mapping will be limited, as the left ventricular wall thickness only measures a few millimeters in rats. Moreover cavitation emissions from the microbubble-laden blood in the ventricles could overwhelm the small signals from the myocardium. Adequate time gating and limited expectations with respect to spatial resolution would make passive cavitation mapping/monitoring an orthogonal therapy feedback in addition to ECG. This approach to treatment management would be investigated for also providing spatial feedback when using a larger animal model for MCET.

Conclusions

Strategies to refine MCET were tested in an effort to make this method amenable to clinical translation. Simulated scanning was as effective as the normal fixed-beam treatment, and high agent infusion allowed reduced treatment duration. Ultrasound path variation and venous catheter placement did not appear to be substantial sources of variability. The LVE

reflected variations in the infused microbubble concentration, but failed to predict efficacy. Comparing suspensions of varied microbubble sizes revealed that LVE was dominated by larger bubbles, while efficacy appeared dependent on smaller sizes. Remaining challenges for realistic clinical translation of MCET include the development of better real time control of the treatment progression, and a characterization of the long term tissue reduction achieved by treatment.

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Figure 1.

The sequence of the triggered pulse-bursts for the square envelope (top) and the Gaussian envelope (bottom). The Gaussian modulation simulates the exposure from a pulsed-focused beam as it is scanned, and produces only 1–2 pulses of the highest amplitudes.

Figure 2. Ultrasound images from the S-10 probe in the exposure position: (a) the heart appears as the circular structure with relatively dark central left ventricle, (b) the left ventricle brightens substantially with contrast agent infusion.

Figure 3.

The analysis of echogenicity from the small oval region in the left ventricle (shown in the small ultrasound image) plotted as acoustical units (AU) versus time. The time-intensity curve varies with the time of the heart cycle, represented by the trace below the plot. At approximate end of diastole, evident in the image and indicated by the red markers in the plot, the AU was about 50 mAU.

Figure 4.

The echogenicity from contrast agent in the left ventricle in acoustical units (AU) plotted against the dose rate of the contrast agent, which shows that the AU was approximately proportional to contrast concentration.

Figure 5.

The acoustic units (AU) of echogenicity from the left ventricle with contrast agent during treatment. The AU decreased greatly to about 1 mAU immediately after a triggered pulse burst, but then recovered with successive heartbeats to about 12 mAU just before the next pulse burst.

Figure 6.

The echogenicity from intervening tissue between the chest surface and the heart, given in acoustical units (AU). The tissue echogenicity was relatively high for lower LVE, which likely resulted from scattering and attenuation by the intervening tissue reducing the ultrasound transmission to and from the left ventricle.

Figure 7.

Left ventricular echogenicity in acoustical units (AU) and efficacy results for the different groups of rats. Group F was a sham and calibration group, as described in the text. The results are presented as the means $\pm$ the standard deviation, with $n=5$ except $n=4$ for group C.

Figure 8.

Results for the microbubble size fractionation experiment with infusion adjusted for constant microbubble concentration. The substitute contrast agent was used in place of Definity for this experiment (see Methods). The small- and large-size enhanced contrast agent suspensions were processed to modify the normal size range, see Fig. 11. Results for the normal suspension are listed for comparison. Abbreviations are: PC premature contractions as a percentage of the number of pulse triggers, AU acoustical units of maximum echogenicity for infusion before beginning treatment, EB Evans blue.

Figure 9.

The stained cell scores (SCS) of individual rats designated by their group membership letter plotted in relation to the observed contrast echogenicity from the left ventricle just before beginning treatment. The SCS outcome of the treatment is approximately independent of the starting agent scattering acoustical units, as indicated by the linear correlation line.

Figure 10.

The stained cell scores (SCS) of individual rats plotted as in Fig. 9 versus the observed contrast echogenicity from the left ventricle after refill, and just before the next triggered pulse burst. The SCS outcome of the treatment is approximately independent of the refill scattering acoustical units, as indicated by the linear correlation line.

Figure 11.

Plots of the microbubble size distributions for the normal contrast agent suspension, and for suspensions processed to enhance the small or large microbubble fraction. The distributions have been normalized for comparison, and the suspensions were diluted as needed to achieve the same infusion concentration. The sizing method included the 1–30 μm size range, but there were very few larger than about 6 μm.

Table 1

The 5 sets of conditions used for the different groups of rats. Group F was a sham and calibration group, as described in the text. For group C, the normal aiming view was changed by vertically moving the scan plane in an effort to simulate poor aiming, which might involve proximity to intervening ribs.

Group	Durations	Infusion $\mu\text{l/kg/min}$	Infusion vein	Modulation envelope
A	300	5	tail	square
B	300	5	jugular	square
C	300	5	jugular	square
D	300	5	jugular	Gaussian
E	120	12.5	jugular	Gaussian