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SUBHARMONIC AIDED PRESSURE ESTIMATION FOR MONITORING INTERSTITIAL FLUID PRESSURE IN TUMORS: CALIBRATION AND TREATMENT WITH PACLITAXEL IN BREAST CANCER XENOGRAFTS

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Abstract

This project noninvasively estimated interstitial fluid pressure (IFP) in rats with breast cancer xenografts using subharmonic aided pressure estimation (SHAPE) versus an invasive pressure monitor. Moreover, monitoring of IFP changes after chemotherapy was assessed. Eighty-nine rats (calibration n=25; treatment n=64) were injected with 5×10⁶ breast cancer cells (MDA-MB-231). RF signals were acquired (39 rats successfully imaged) with a Sonix RP scanner (BK Ultrasound, Richmond, BC, Canada) using a linear array (L9–4; transmit/receive: 8/4MHz) after administration of Definity (Lantheus Medical Imaging, N Billerica, MA; 180µl/kg) and compared to an invasive pressure monitor (Stryker, Berkshire, UK). An inverse linear relationship was established between tumor IFP and SHAPE ($y = -1.06x + 28.27$; $r = -0.69$; $p = 0.01$) in the calibration group. Using this relationship in the treatment group resulted in $r = 0.74$ ($p < 0.05$) between measured (pressure monitor) and SHAPE-estimated IFP (average error 6.24mmHg). No significant differences were seen before/after paclitaxel treatment (5mg/kg, Mayne Pharma, Paramus, NJ) with either method ($p = 0.15$).

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Keywords

Subharmonic-Aided-Pressure-Estimation; Ultrasound; Interstitial-Fluid-Pressure; Contrast Agents; Microbubbles; Breast Cancer Xenografts

Introduction

Locally advanced breast cancer (LABC) is the designation for stage II or III breast cancers, where the tumor has not metastasized to parts of the body other than the breast and axillary lymph nodes (Favret et al. 2001, Kaufmann, et al. 2003). Neoadjuvant chemotherapy (systemic preoperative therapy) is the standard of care for the treatment of LABC (Esteva & Hortobagyi 1998, Guarneri et al. 2007). Studies have shown that it is as efficient as adjuvant chemotherapy (postoperative therapy) when assessed by disease-free survival (at 9 years 53% in adjuvant, 55% in neoadjuvant, $p=0.5$) and overall survival (at 9 years 70% in adjuvant, 69% in neoadjuvant, $p=0.8$) (Wolmark et al. 2001). In addition, it offers considerable benefits to patients, such as relatively reduced tumor size at the time of surgery and a greater chance of breast conservation and reconstruction (Guarneri et al. 2007, Mauri et al. 2005, McMasters & Hunt 1999, Waljee & Newman 2007). By monitoring treatment, it may be possible to predict patient response to chemotherapy early on and thereby to optimize the treatment (Guarneri et al. 2007, McMasters & Hunt 1999). One of the treatment indicators suggested is the interstitial fluid pressure (IFP) in breast cancers that could potentially be used to monitor the response to neoadjuvant chemotherapy (Taghian et al. 2005).

Generally, the IFP is 10–30 mmHg higher in solid tumors than in normal tissue although values of up to 60 mmHg have been recorded (Halldorsdottir et al. 2014, Heldin et al. 2004, Less et al. 1992). Similarly, IFP in breast cancers has been shown to be higher than that of the surrounding normal breast tissue (Less et al. 1992). This increase is believed to be due to poor lymphatic drainage, leaky neovascularity and differences in the composition of the interstitial matrix in tumors, and it can result in poor transport of therapeutic drugs to tumors (Heldin et al. 2004). Taghian and colleagues (2005) employed a wick-in-needle technique to monitor the IFP of breast cancer before and after neoadjuvant chemotherapy with two different chemotherapy drugs used consecutively. When used as a first drug, paclitaxel decreased the IFP by 36 % ($p = 0.02$), while with doxorubicin as a first drug there was no significant difference ($p = 0.52$). However, tumor IFP measurements have been shown to predict disease free survival for cervical cancer (Milosevic et al. 2001). Currently, the reference standard for IFP measurements is an invasive wick-in-needle method, and thus, an improved technique for measuring IFP is required if this treatment indicator is to become useful in clinical practice.

Vascular ultrasound contrast agents (UCA) are gas-filled microbubbles (diameter generally less than 8 μ m) that improve the depiction of vascularity in ultrasound (US) images by enhancing the difference in reflectivity between tissue and the UCA (Wheatley 2001). Given the difference in compressibility between the surrounding medium and a gas microbubble, any changes in hydrostatic pressure induce changes in the size of the microbubble, which in

turn affects the reflectivity and resonance frequency of the bubble (Ishihara et al. 1988, Shi et al. 1999). Furthermore, UCA can act as nonlinear oscillators producing higher and lower harmonics in the received signals (Goldberg et al. 1994, Kono & Mattrey 2001). In subharmonic imaging (SHI) pulses are transmitted at a frequency f_0 and the echoes are received at half that frequency ($f_0/2$) (Forsberg et al. 2000, Shi et al. 1999).

Our group has developed a novel technique, called subharmonic aided pressure estimation (SHAPE), utilizing the subharmonic amplitude of the scattered signal from the microbubbles for pressure tracking (Shi et al. 1999, Halldorsdottir et al. 2011, 2014, Eisenbrey et al. 2013, Dave et al. 2013). It was demonstrated *in vitro* and *in vivo* that the relationship between subharmonic amplitude and acoustic output can be described by a characteristic sigmoidal curve (Shi et al. 1999, Halldorsdottir et al. 2011, Eisenbrey et al. 2011) with three different stages of subharmonic generation depending on the acoustic output i.e., occurrence, growth and saturation. The occurrence and saturation stages are not favorable for pressure estimation, but in the growth stage there is an inverse linear relationship between the hydrostatic pressure and the subharmonic amplitude (Shi et al. 1999, Halldorsdottir et al. 2011). This inverse linear relationship has been established for several UCAs, over different frequencies (2.5 – 6.6 MHz) and acoustic outputs (0.35 – 0.60 MPa) showing a decrease of 10–14 dB over a pressure range of 0 to 186 mmHg ($r^2 > 0.97$, $p < 0.05$; Halldorsdottir et al. 2011).). Moreover, an *in vivo* proof of concept for estimating the aortic pressure in dogs has been performed (Forsberg et al. 2005). Our group has also looked at a variety of pressure estimation applications *in vivo* (Dave et al. 2012a, 2012b, Eisenbrey et al. 2011, 2013, Forsberg et al. 2016).

We hypothesize that SHAPE can be used to estimate the IFP in LABC tumors, thereby allowing the response to chemotherapy to be monitored. In this paper, we first calibrated the SHAPE method on a Sonix RP scanner using nude, athymic rats with breast cancer xenografts and then applied these calibration curves to an independent data set using different rats with breast cancer xenografts undergoing chemotherapy with paclitaxel.

Materials and Methods

Modification of US Scanner

Based on previous *in vitro* water-tank and *in vivo* proof of concept studies by our group (Halldorsdottir et al. 2011, 2014) a Sonix RP scanner (BK Ultrasound, Richmond, BC, Canada; Software version 3.2.2) was modified by BK Ultrasound and LBJ Development (Fort Mitchell, KY) to enable subharmonic amplitude detection and display in real time for SHAPE.

The ultimate goal of this project is to use SHAPE on LABC in patients and so a high frequency linear array probe L9–4/38 was selected for scanning, as its bandwidth (4 to 9 MHz; center frequency 5.5 MHz) is suitable for breast imaging. An 8.0 MHz transmit frequency and 4.0 MHz receive frequency were implemented on the Sonix RP system for pressure estimation. Moreover, pulse inversion was implemented to minimize linear signal components. A dual screen display, with a B-mode image on one side and a SHI image on the other side was also implemented; similar to previous work by our group (Eisenbrey et al.

2011). Moreover, a feature allowing for the optimization of the acoustic output for SHAPE was added. A graph of the subharmonic amplitude vs. all possible acoustic outputs was generated (Figure 1) and the acoustic output, corresponding to the subharmonic response most sensitive to ambient pressure changes was selected and used to acquire pressure measurements. This is in part based on a solution previously proposed by our group (Dave et al. 2013).

Cell Culture

MDA-MB-231 breast cancer cells (from Dr. Lanza-Jacoby at Thomas Jefferson University, Philadelphia, PA) were cultured (with 89 % DMEM, 10% fetal bovine serum, and 1% penicillin streptomycin) and stored in an incubator (set at 37°C and 5.0% CO₂). All cell culture materials were supplied by Fisher Scientific (Waltham, MA). Cells were implanted subcutaneously together with growth factor reduced Matrigel basement membrane matrix purchased from BD Biosciences (San Jose, CA).

Animal model

Eighty nine athymic, nude, female, rats (100–120 g, 6 weeks old at cell injection, NIH-RNU-F, Taconic, Hudson, NY) were randomized into either a calibration group (25 rats) or a treatment group (64 rats). Subcutaneous injections of 5×10^6 MDA-MB-231 tumor cells into the right mammary fat pad of each rat were performed. After injections the animals were returned to the cage, given food and water *ad libitum*. Tumor growth was inspected on days 7, 14, and 21 and tumor dimensions measured with a caliper once tumors had formed.

Tumor Scanning

The rats were anesthetized and received 3 bolus intravenous (IV) tail vein injections of the UCA Definity (180 µl/kg; Lantheus Medical Imaging, N Billerica, MA) followed by a 0.2 ml saline flush. First, SHI was performed with the modified Sonix RP scanner and the optimal acoustic output established. Then, the IFP of the normal mammary fat pad (n = 3) as well as the tumor (n = 3) was measured with an invasive needle based intra-compartmental Stryker pressure monitor (as the IFP reference standard). SHAPE data was collected for 2 s for each measurement at a scanning depth of 4 cm and a frame rate 15 Hz. Focus placement was dependent on depth and tumor location. Concurrently with the IFP measurements, SHAPE measurements were acquired (n = 3) at the optimal acoustic output setting and averaged for each SHAPE data point.

For the treatment group the ability of SHAPE to monitor changes in IFP before and after administration of the chemotherapy agent paclitaxel (Mayne Pharma, Paramus, NJ) was tested. SHAPE and the Stryker pressure monitor measurements were performed before (on day 21, 24 or 28) and 48 hours after administration of a single IV injection in a lateral tail vein of 5 mg/kg of paclitaxel. The paclitaxel dosage (5 mg/kg) was selected based on a study by Shord and Camp (2006). Additionally, tumor volumes were measured with a caliper every other day to allow growth curves to be established and compared.

Data Processing and Statistical Analysis

The RF data acquired were transferred to a PC for offline analysis. A fast Fourier transform (FFT) was calculated for each vector in each frame and the values converted into logarithmic form (i.e., in dB) using Matlab (version R2014b; Mathworks, Natick, MA). A maximum projection image (MPI) was generated and a region of interest (ROI) selected containing normal tissue and tumor. The size of the ROI was selected such that the whole tumor could be assessed as well as surrounding tissue. For each ROI thresholding was performed by calculating the mean pixel value (MPV) for all frames combined (MPV_{all}) and for each frame separately (MPV_{frame}). For each frame, the MPV_{frame} was then compared to three different thresholds corresponding to 1.00x, 1.15x, 1.30x MPV_{all} and an average of all pixel values above the threshold in that frame was calculated. The results for all the frames were then averaged over 2 s (Figure 2). The threshold values were selected based on previous work by our group (Eisenbrey et al. 2011).

The relationship between microbubble based subharmonic signals and the IFP measured with the intra-compartmental monitor was established (essentially calibrated) using linear regression analysis. Calibration equations were generated and applied to the independent data set from the treatment phase of the study. Analysis of variance (ANOVA) was used to compare the 3 different thresholds. This analysis was then repeated with treatment (control/paclitaxel, pre/post treatment) using linear regression, ANOVA and paired t-test as applicable to establish if any interactions occurred. All statistical analyses were conducted using Stata 9.0 (Stata Corporation, College Station, TX) and Matlab with p-values less than 0.05 indicating significance.

Results

Calibration Group

Of the 25 rats implanted with MDA-MB-231 for the initial calibration studies, 16 (64%) exhibited tumor growth and 13 (52%) were successfully imaged, whereas technical difficulties prevented imaging of 3 rats. An example of the ROI selection and data acquisition process, starting with an MPI SHI grayscale image and showing the impact of the three thresholds (100, 115 and 130%) can be seen in Figure 3. Notice, how at the 100% threshold (Fig 3c) there is still some tissue signal remaining whereas at the 130% threshold (Fig 3e) some of the vasculature is not included.

An inverse linear relationship was confirmed for all thresholds with the highest correlation at the 115% threshold (r : -0.64 for 100%, -0.69 for 115% and 0.61 for 130%, $n=25$, $p < 0.01$). As the Stryker pressure monitor is sensitive to movement and the angle of insertion, three data points where the standard deviation was larger than 5 mmHg were removed. The best fit calibration equation at the 115% threshold was:

$$IFP = \frac{SHAPE - 127.221}{-1.0624} \quad (1)$$

This relationship is presented in figure 4.

The three threshold groups were all significantly different from each other ($p < 0.01$) and the difference between each group (100% and 115%, 100% and 130%, 115% and 130%) was also statistically significant ($p < 0.01$). A box plot of the subharmonic data per threshold is shown in Figure 5. The mean subharmonic amplitude for 100%, 115% and 130% thresholds was 111.20 ± 10.59 dB, 119.70 ± 12.06 dB and 136.51 ± 12.77 dB, respectively.

The average difference between tumor IFP and tissue IFP was 13.26 ± 6.70 mmHg and the average difference in subharmonic amplitude between tumor and tissue was -13.40 ± 5.25 dB (100%), -14.45 ± 5.59 dB (115%) and -15.47 ± 6.71 dB (130%). When comparing the change in subharmonic amplitude between tumor and tissue and the change in IFP between tumor and tissue for the calibration rats ($n = 13$) using linear regression analysis no significant relationship was seen ($p > 0.35$).

Verification of Calibration with an Independent Data Set

For the treatment groups, 64 rats received injections of the breast cancer cells, which resulted in 38 animals with tumors (59%) and amongst those 26 (41%) were successfully imaged. The 12 rats with tumor growth that failed the imaging study were mainly for technical reasons. Six rats (2 each on day 21, 24 or 28) were designated as control cases (i.e., they did not receive injections of paclitaxel).

The treatment results were used as an independent data set to test whether the calibration equations could be used to accurately estimate the pressure differential between the tumor and surrounding tissue (Figure 6). To better simulate a clinical situation, all values with calculated IFP below -10 mmHg were removed (9, 13, 13 points removed for 100%, 115% and 130% respectively). The measured and calculated IFP for 100% and 130% thresholds were significantly different ($p = 0.01$ for both groups) but not so for 115% threshold ($p > 0.13$). This further indicates that the 100% and 130% thresholds are less suitable than the 115% threshold for SHAPE IFP estimation. The correlation coefficient ranged from 0.73 to 0.74 and errors ranged from 7.80 mmHg to 8.55 mmHg (Table 1). Moreover, a paired t-test showed that the measured and calculated groups were not significantly different ($p > 0.14$).

Treatment group

For the treatment studies, an inverse linear relationship was established for all three thresholds ($r: -0.72$ to -0.74 ; $n = 102$; $p < 0.01$). This is a higher correlation than was seen in the calibration study ($r: -0.61$ to -0.69) and is likely due to the increased number of rats. However, the difference in correlation coefficients was not significant ($p > 0.25$). The highest correlation was at 100% ($r = -0.74$, $p < 0.01$). Moreover, the correlation for all three thresholds was equivalent ($p > 0.76$). A best fit plot for the 100% threshold is shown in Figure 7. As expected from the calibration phase of the study, all three thresholds were significantly different including between groups ($p < 0.01$). A box plot of the subharmonic data per threshold is presented in Figure 8. The mean for the subharmonic amplitude was 110 ± 11.7 dB, 122.0 ± 12.4 dB and 135.5 ± 14.4 dB for 100%, 115% and 130% threshold levels, respectively.

The slopes and intercepts of the calibration equations were compared to the slopes and intercepts of the treatment equations to estimate whether a universal model could be derived.

No significant differences were seen and therefore a universal equation could potentially be derived for future pressure estimation. This is further supported by the fact that there was no statistically significant difference ($p > 0.14$) between the calculated and measured IFP at 115% threshold in the calibration phase. Linear regression analysis was used to investigate the subharmonic data depending on the day of treatment (i.e., day 21, 24 or 28). An inverse linear relationship was seen in all cases (r : -0.63 to -0.78 ; $n = 34$; $p < 0.01$). The highest correlation was found for the 115% threshold on day 28 ($r = -0.78$).

The average difference in IFP between tumor and tissue was 13.97 ± 6.72 mmHg and the average difference in subharmonic amplitude between tumor and tissue was: -16.49 ± 9.62 dB (100%), -18.76 ± 8.33 dB (115%) and -20.29 ± 9.97 dB (130%). Note, that $n = 51$ as there are 26 rats and for each rat both tumor and tissue IFP were measured pre and post treatment except in one rat where no post measurement was taken. No significant relationship was seen for the treatment group when looking at the difference in subharmonic amplitude between tumor and tissue and comparing it to the difference in IFP between tumor and tissue ($p > 0.15$). Furthermore, there was no significant difference between the treatment and control groups at any threshold ($p > 0.6$; data not shown).

No significant difference in tumor IFP was seen between the rats before and after treatment with tumor IFP decreasing from 15.3 ± 7.0 mmHg to 12.6 ± 6.2 mmHg after the paclitaxel treatment ($p = 0.15$). Likewise, the difference in subharmonic amplitude pre and post treatment when compared to difference in IFP pre and post treatment was not significant ($p > 0.35$). The difference in IFP from pre to post treatment for each rat in both the control and treatment groups is depicted in Figure 9. Essentially, there was no difference between IFP before and after treatment with paclitaxel and thus, no treatment response for SHAPE to monitor.

The average level of IFP seen in the rat tumors was 14.0 ± 6.7 mmHg. No statistical difference ($p = 0.98$) in tumor IFP was seen depending on which day the pressure was measured with averages of 8.9 ± 9.4 mmHg (day 21), 7.1 ± 7.9 mmHg (day 24) and 8.0 ± 8.4 mmHg (day 28), respectively.

Discussion and Conclusion

SHAPE has been evaluated as a tool for IFP estimation in breast cancer during neoadjuvant chemotherapy. This is the first study in which SHAPE has been investigated *in vivo* at frequencies higher than 5 MHz and the first using SHAPE in angiogenic neovessels.

Previous studies conducted by our group have established an inverse linear relationship for several UCAs, over different frequencies and acoustic outputs showing a decrease of (Halldorsdottir et al. 2011). These studies indicate that the method is dependent on the contrast agent used as well as the frequency and acoustic output. Definity was selected for this study for four main reasons: 1) it is commercially available in the United States, 2) it is approved by the FDA for echocardiography use in the United States and finally 3) our previous *in vitro* studies on lower frequency (< 7 MHz) SHAPE in a water-tank showed up to 13 dB decrease ($r^2 = 0.98$, $p < 0.05$) in subharmonic amplitude for Definity at 6.6 MHz

transmit frequency when increasing the hydrostatic pressure from 0 to 186 mmHg, thereby giving promise for higher frequencies and 4) further *in vitro* optimization studies using a Sonix RP scanner at transmission frequencies of 6.7 MHz and 10 MHz (receiving the subharmonic at 3.35 MHz and 5 MHz respectively) and over a range of acoustic outputs showed that the greatest sensitivity to pressure estimation was seen at 10 MHz and -8 dB ($r^2 = 0.95$; $p < 0.01$). We have only studied two tumor models; breast cancer and melanoma in swine, using a similar protocol and acoustic parameters. Both tumor models are superficial and as such require similar frequencies. One can speculate that different tumor models would require calibration as well as different acoustic parameters (frequency, acoustic output) are needed depending on their location in the body.

The highest correlation in the calibration group ($r = -0.69$) was achieved at a 115% threshold. From this linear regression analysis, we obtained calibration equations that were then applied to an independent data set from the treatment phase of the study to generate calculated IFP values that were then compared to the measured IFP values from the same data set. Absolute errors ranged from 7.80 mmHg to 8.55 mmHg and root mean square errors ranged from 7.49 mmHg to 10.78 mmHg, which is on par with previous work by our group in a flow phantom where absolute errors and RMS errors were 6.70 mmHg and 8.16 mmHg, respectively (Dave et al. 2012a). The Stryker pressure monitor used, is very sensitive to the angle of insertion and introduces some measurement error. This is controllable by calibrating the pressure monitor as per the manufacturer's instructions before each measurement set and keeping it steady for the duration. Batch to batch variability of Definity might also contribute to a small degree, although this cannot be controlled. As was expected from the calibration study an inverse linear relationship was observed between the subharmonic amplitude and the IFP for all three thresholds ($r: -0.72$ to -0.74 , $p < 0.01$, $n = 104$) this is consistent with results from previous studies (Dave et al. 2012a, 2012b, 2012c, 2013, Halldorsdottir et al 2011, Shi et al. 1999).

All thresholds were significantly different from each other. This is consistent with the results of the calibration study and it should be noted that the intercepts and slopes for the equations derived for calibration are very similar to the ones from the treatment study. This suggests that a calibration curve might be used to estimate IFP at least when using the same agent, scanner and acoustic parameters for the same tumor type at a relatively uniform and shallow depth (< 4 cm).

The current gold standard for IFP measurements is the wick-in-needle technique, where a system based on a needle filled with a nylon wick or a fiberoptic pressure transducer is used to measure IFP. This method has been applied in a number of studies (Curti et al. 1993, Fadnes et al. 1977, Heldin et al. 2004, Lunt et al. 2008, Ozerdem & Hargens 2005, Ozerdem 2009, Simonsen et al. 2012); most notably by Taghian et al. (2005) that used the wick in needle to monitor IFP treatment response to paclitaxel and doxorubicin in breast cancers. As that was a hypothesis-generating study, the investigators did not demonstrate any outcome related to the relationship between IFP and therapy response (Taghian, et al. 2005). Another method that has been studied for IFP measurements is micropuncture using glass capillaries; this method is accurate, but only practical for superficial tumors as measurements are limited

to 1 mm below the surface (Heldin et al. 2004). SHAPE offers the benefit over both these methods of being noninvasive.

There was no statistical difference in subharmonic amplitude between the control and treatment groups, due to a lack of treatment effect from the low paclitaxel dose used (5 mg/kg). Moreover, there were no significant changes in IFP between days and no significant changes pre to post treatment. Paclitaxel is more toxic to rats than to humans and the dose given was only 25% of what is normally used in humans (5 vs. 20 mg/kg) as that is the highest safe paclitaxel dose for rats (Shord & Camp 2006). This might also have contributed to the lack of effect from the chemotherapy. Thus, it is impossible to make any conclusions on whether or not SHAPE is sensitive enough to estimate changes in IFP for treatment monitoring based on this study.

The average level of IFP seen in the rat tumors was 14.0 mmHg which is comparable to tumor IFP levels seen by Ferretti et al. in a rat tumor model with BN472 breast cancer xenografts (Ferretti et al. 2009), but considerably higher than what we expected to see from human studies as Taghian et al. (2005) saw a mean level of 6.9 mmHg but a similar decrease (2.5 mmHg and 2.7 mmHg) in tumor IFP after paclitaxel treatment.

Currently, a clinical study on SHI in human breast cancer is underway and IFP estimation is part of that investigation, building on the results of this study using a Logiq 9 scanner with encouraging results indicating that SHAPE IFP tumor estimates could become an additional marker in the monitoring of neoadjuvant chemotherapy in breast cancer (Forsberg et al. 2016; Nam et al. 2016).

In conclusion, results from this study demonstrate that SHAPE may be useful for the noninvasive monitoring of IFP. Moreover, it would make it easier to customize individual patient treatment if SHAPE was found to be able to monitor neoadjuvant treatment response throughout the chemotherapy cycles and early clinical results are encouraging (Nam et al. 2016) showing reasonable agreement with this study.

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Figure 1.

An example of the acoustic output curve generated by the modified solution. The green arrow points to the data point selected for scanning. Note the S shape of the curve corresponding to theory.

Figure 2.
Block diagram of the off-line processing performed on the RF data.

Figure 3. Breast tumor xenograft (arrows) depicted in maximum projection intensity (MIP) SHI mode (a) and the same tumor using a different color scheme for clarity (b). The ROI selected from image (b) is then shown for 100% (c), 115% (d) and 130% (e) thresholds.

Figure 4.

Subharmonic amplitude results compared to the pressure monitor at a 115% threshold. In this case data points with a IFP standard deviation larger than 5 mmHg have been eliminated. Note that for a clearer comparison relative values for subharmonic amplitude (0 dB corresponding to the lowest dB value and then subharmonic amplitude difference relative to that value) are reported.

Figure 5. Box plot of the subharmonic data per threshold level. All groups are significantly different from each other. Red line indicates the median, blue box indicates first and third quartile.

Figure 6.

Comparison for the 115% threshold of calculated IFP from calibration equations where values below -10 mmHg have been removed and SHAPE data from the treatment phase of the study and the corresponding IFP measured during data acquisition.

Figure 7. Subharmonic amplitude results compared to the pressure monitor at a 100% threshold. Note that for a clearer comparison relative values for subharmonic amplitude (0 dB corresponding to the lowest dB value and then subharmonic amplitude difference relative to that value) are reported.

Figure 8.

Box plot of the subharmonic data per threshold. All groups are significantly different from each other. Red line indicates the median, blue box indicates first and third quartile. Outliers are beyond $\pm 2.7\sigma$.

Figure 9.
The difference in IFP from pre to post treatment for each rat in both the control and treatment groups

Table 1

Comparison of measured and calculated IFP values in the treatment group.

Threshold	r value, p value	Absolute Error [mmHg]	RMS error [mmHg]	p value from t test	n
100%	r = 0.73, p < 0.01	8.00	9.42	0.01	95
115%	r = 0.74, p < 0.01	6.24	7.49	0.91	91
130%	r = 0.73, p < 0.01	7.75	9.20	0.01	91