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Improved Sonothrombolysis from a Modified Diagnostic Transducer Delivering Impulses Containing a Longer Pulse Duration

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Abstract

Although guided high mechanical index (MI) impulses from a diagnostic ultrasound transducer have been used in pre-clinical studies to dissolve coronary arterial and microvascular thrombi in the presence of intravenously infused microbubbles, it is possible that a longer pulse duration (PD) than that used for diagnostic imaging may further improve the effectiveness of this approach. Using an established in vitro model flow system, a total of 90 occlusive porcine arterial thrombi (thrombus age 3–4 hours) within a vascular mimicking system were randomized to 10 minute treatments with two different PD (5 μsec and 20 μsec) using a Philips S5-1 transducer (1.6 Megahertz center frequency) at a range of MI's (from 0.2 to 1.4). All impulses were delivered in an intermittent fashion to permit microbubble replenishment within the thrombosed vessel. Diluted lipid-encapsulated microbubbles (0.5% Definity®) were infused during the entire treatment period. A tissue mimicking phantom of 5 cm thickness was placed between the transducer and thrombosed vessel to mimic transthoracic attenuation. Two 20 MHz passive cavitation detection systems (PCDs) were placed confocal to the insonified vessel to assess for inertial cavitational activity. Percentage thrombus dissolution (% TD) was calculated by weighing the thrombi before and after each treatment. %TD was significantly higher with a 20 μsec PD already at the 0.2 and 0.4 MI therapeutic impulses (54±12% vs. 33±17% and 54±22% vs. 34±17%, $p<0.05$ compared to 5 μsec PD group, respectively), and where PCD's detected only low intensities of inertial cavitation. At higher MI settings and 20 μsec PD, %TD decreased most likely from high intensity cavitation shielding of the thrombus. Slightly prolonging the PD on a diagnostic transducer improves the degree of sonothrombolysis that can be achieved without fibrinolytic agents at a lower mechanical index.

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Keywords

Thrombolysis; Imaging; Ultrasound

Introduction

Although guided high mechanical index (MI) impulses from a diagnostic ultrasound transducer have been useful in pre-clinical studies in dissolving arterial and microvascular thrombi in the presence of intravenously infused microbubbles (Xie et al. 2009a; Xie et al. 2009b), there is concern that these high MI impulse may also have a detrimental effect. The degree of damage within tissue is directly related to the applied peak negative pressure (PNP) of the ultrasound pulses (Hwang et al. 2005). Since microbubbles serve as a nucleus for cavitation, the acoustic pressure threshold required to induce a specific cavitation state is lowered when they are present within the field of insonation (Deng et al. 1996; Prokop et al. 2007). Cavitation is generally classified into two types, stable cavitation (SC) (Miller 1988), which results in emissions at sub- and ultra-harmonics of the main excitation frequency, and inertial cavitation (IC)(Crum 1988; Bailey et al. 1999), which is characterized by broadband noise emissions. These cavitational responses can be elicited from commercially available microbubbles when insonified with diagnostic ultrasound frequencies and mechanical indices (Xie et al. 2009a). However, diagnostic ultrasound pulse durations are very short, and all previous work with diagnostic ultrasound transducer frequencies to enhance thrombus dissolution have been in the presence of TPA with or without microbubbles (Laing et al. 2012; Ricci et al. 2012; Basta et al. 2004; Xie et al. 2013). There is no data on the use of diagnostic ultrasound and microbubbles alone (without a fibrinolytic agent) to dissolve thrombi. Part of the reason for this is that longer pulse durations from single element non-imaging transducers have been shown to be more effective at sustaining the cavitational response, and in improving microvascular thrombus dissolution (Leeman et al. 2012). Although these pulse durations were beyond what could be achieved with a conventional diagnostic ultrasound transducer, modifications can be made in diagnostic transducers to slightly prolong pulse duration (PD) at the expense of resolution. In this study, we modified a diagnostic transducer such that it could prolong the PD while still keeping total output within Food and Drug Administration limits. We hypothesized that prolonging the PD on a diagnostic transducer (and hence the duration of cavitation) may be a method of reducing the MI required to achieve primary ultrasound fibrinolysis. Such a modification would result in significant new therapeutic applications for diagnostic systems both in the setting in acute coronary syndromes as well as stroke, and lessen, or even eliminate, the need for fibrinolytic therapy.

Materials and Methods

Experimental apparatus

The overall experimental arrangement consisted of large acrylic tank (100 cm×50 cm×40 cm) filled with distilled deionized water described previously (Xie et al. 2011). The set-up includes a flow system, a five centimeter thick tissue-mimicking phantom (TMP) with an

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adaptor module, transducer positioning system, data display, and collecting and analysis system, along with a two-dimensional ultrasound system.

Flow within the tubing (2.6 mm internal diameter) was controlled by a Master-flex flow pump (Cole Parmer Instrument Co., Vernon Hills, IL, USA). Phosphate-buffered saline (PBS) in a large beaker was maintained at 37°C using an Isotemp digital stirring hotplate (Fisher Scientific; Dubuque, IA, USA), which was then infused through the tube system at a constant flow rate of 20 mL/min. This rate simulates the flow rate of human or pig mid left anterior descending coronary artery in an underperfused state (Hildick-Smith and Shapiro 2000; Ootaki et al. 2005). A bypass-vessel was aligned in parallel to the thrombosed vessel to prevent the high pressure produced by thrombotic occlusion (Figure 1). The bypass-vessel and thrombosed vessel (T-connector) were kept at the same level to ensure equal pressure in the flow system. The tube system was then placed under a five centimeter thick tissue-mimicking phantom (TMP) designed by Computerized Imaging References Systems, Inc. (Norfolk, VA, USA) in order to simulate transthoracic imaging. According to the manufacturer, the TMP attenuates ultrasound at 0.49 dB/cm/MHz. The base of the adaptor module had an eight by eight centimeter cut-out that was used for exchange of thrombi within the T-shaped connectors. An epoxy holder was extended from the edge of the hole to hold the T-connector in place. Plastic clips on the module were used to lock the extension tubes. To exchange the T-connector for each experimental run, the extension tubes were released and the exchange procedure performed above water to prevent microbubble leak into the water tank.

The ultrasound transducer (S5-1; iE 33; Philips Healthcare) was held in position by a clamp holder attached to a ringstand. It was mechanically adjusted left or right to align the beam over the thrombus. The Positioning System was used to confocally align the transducer beam with the passive cavitation detectors.

Arterial vascular access and clot preparation

Arterial whole blood samples were obtained from a healthy pig. The blood withdrawal procedures were compliant with the Guidelines of the Institutional Animal Care and Use Committee (IACUC) and the Standards in the Guide for the Care and Use of Laboratory Animals. An arterial vascular access catheter was placed in the right carotid artery and tunneled subcutaneously to the dorsal region of the chest wall. On the day of the experiment, a 2.0-mL sample was collected and 3 IU/mL thrombin (Sigma Aldrich, Milwaukee, WI, USA) added into the blood. This dose of thrombin was chosen as it was the minimum dose required to produce adherent thrombi at three hours. A 40-μL sample of this thrombin-whole blood mixture was injected into the T- connectors. These samples were then allowed to stand at room temperature (22°C) for three hours prior to being placed in the set-up described above.

Microbubbles

Definity, a lipid-encapsulated commercially available microbubble (Lantheus Medical Imaging, N. Billerica, MA, USA) was used for all experiments. The mean diameter size of these microbubbles is 1.1 –3.3 μm, and concentration 1.2×10^{10} microbubbles/mL. During

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the experiment, 0.5 mL Definity was added in 100 mL normal saline and then infused at 1 mL/min via a stopcock into the flow system containing PBS. This resulted in approximately 6×10^7 microbubbles per minute flowing through the connector/bypass system displayed in Figure 1.

Modified Two Dimensional Ultrasound System

The iE33 diagnostic scanner was a two-dimensional (2D) sector array with a center frequency of 1.6 MHz. The system was modified for image-guided sonothrombolysis capability. The specific modifications included: (a) a therapeutic ultrasound (TUS) mode with a pre-defined treatment area (similar to the box in color Doppler imaging) superimposed on the anatomic imaging mode (B-mode); and (b) a low MI contrast-only imaging mode for monitoring the presence (replenishment) of microbubbles. For each TUS frame, 19 equally spaced beams (each with an ensemble of four 1.6MHz long pulses) were scanned into a 54 degree angular sector area. The beams were focused at the center of the thrombosed vessel. The frame rate for the TUS mode was set to be 50 Hz. The two TUS modes tested were a 20 μsec and 5 μsec pulse duration, and transmitted at MI values that were achievable with current diagnostic scanners. The acoustic energy for the imaging pulses in the background B-mode was much less that for the two TUS settings. For a typical moderate TUS setting (e.g., four 20 us pulses at each direction with MI=0.6), the TUS intensity is approximately 4500 times that for a typical low MI imaging setting (e.g., short pulses with very low MI=0.1). Even at the lowest TUS power setting (5us pulses with MI=0.2), the ultrasound intensity for TUS is still approximately 30 times that for the low-MI imaging setting.

Experimental Protocols

A 0.5 % infusion of microbubbles (Definity; Lantheus Inc.) at 1 ml/min was continuously administered, and mixed with an infusion of PBS at 37°C, so that actual concentration of microbubbles reaching the thrombus region was 6×10^7 microbubbles/minute. Treatments were randomized to two different pulse durations: 5 μsec or 20 μsec. The MI for each group was gradually adjusted from 0.0 to 1.4. All impulses were delivered in an intermittent fashion to permit microbubble replenishment within the thrombosed vessel. Total treatment time was 10 minutes. Six samples were tested for each of the different MI settings in each PD group. Six measurements of thrombus area change over the same 10 minute time period were also determined with microbubbles alone in the absence of ultrasound.

Before testing, each thrombus sample was placed and weighed on a balance with 0.001g resolution (Adam Equipment Inc., Milton Keynes, England). After treatment, the sample was first imaged using a conventional digital camera (D90, Nikon, Tokyo, Japan) at multiple angles to examine the shape of any residual thrombus within the vessel. The degree of thrombus dissolution was then categorized by a blinded reviewer (JW) as either transmural (no residual thrombus left across the extent of the vessel), heterogeneous (fragments of thrombi noted across the vessel diameter), or asymmetric (residual thrombus noted only along one side of the vessel). After camera images were obtained, the thrombus was recovered, and dried by a 30-minute air blow as described previously (Xie et al. 2011). It

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was then weighed on the same balance. Percent thrombus dissolution (%TD) was calculated by comparison of clot mass before and after treatment.

Cavitation measurements

A computer-controlled three-axis positioner (Velmex® Unislide™) was mounted on the tank. This positioner could locate the co-focus of a pair of 20 MHz single-crystal ultrasound transducers (Olympus-IMS, Waltham, MA) with 10 μm spatial resolution. The transducers, whose 1-mm-diameter focused beams cross at right angles, served as detectors of inertial cavitation occurring at their co-focus by passively recording the 20 MHz components of bubble collapse acoustic emissions. Each transducer's output signal was amplified, filtered, and displayed on a different trace of the digital oscilloscope. A data acquisition program, written in Matlab®, collected the data from a confocal volume within the lumen of the tubing and recorded acoustic signals only from the cavitation signals that occurred in both channels simultaneously. Triggering of the oscilloscope by a custom-designed output signal from the Philips iE33 scanner assured that the only cavitation signals resulting from particular TUS beams scanning across the tubing were being recorded. Both SC and IC co-exist at moderate MI's, as indicated in our previous studies (Vignon et al. 2013). Because the 5us pulse is not sufficiently long for spectral extraction of sub- or ultra-harmonics, we could not compare the SC effects from the 5us and 20us. Therefore a PCD was used only for the direct IC comparisons in this particular study.

Measurements within the lumen were made in the presence of and in the absence of microbubbles, and using the five centimeter thick tissue mimicking phantom. Separate measurements were also made in conditions to simulate the low flow conditions within a thrombus, using very low flow conditions (1 and 5 ml/min) as well as at 20 ml/min.

Statistical Comparisons

All data were expressed as mean ± SD values or number and percentages. Paired t testing was used for changes in thrombus mass before and after each randomized treatment. Analysis of variance was used for comparison of different treatment combinations. A Fisher Exact test was used to compare differences in the appearance of residual thrombus after treatment. Based on power calculations which estimated a 50% thrombus dissolution for a 20 usec PD and 30% thrombus dissolution for a 5 usec PD at any given MI, six samples were tested for each group. This resulted in a power value of 0.90 (Statistical Solution, LLC; www.statisticalsolutions.net). A probability value <0.05 was considered statistically significant.

Results

Thrombus Dissolution Patterns

A total of 90 porcine arterial thrombi were tested with 5 μsec or 20 μsec pulse durations (total of 15 groups getting between 0.2 and 1.4 MI settings at the two different pulse durations, and one group receiving microbubbles alone without ultrasound). Figure 2 demonstrates low MI images and digital images of a thrombus before and after treatment with 20us pulse duration TUS at an MI of 0.6. Prior to treatment, the thrombus appears to

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totally occlude the vessel, but the 0.1 MI imaging mode consistently detected micro-channels within the thrombus during the 10 second off period. In this particular case, at the end of the 10 minute treatment (lower panels), there is transmural clearance of the thrombus, and the 0.1 MI imaging mode now detects near complete recanalization. We did measure temperature changes with a calibrated thermometer (Fisher Scientific, Dubuque, IA, USA) placed within the flow system of the tubing during application of the mechanical indices (0.2–1.4) at the two pulse durations. No temperature change was recorded during these applications.

Thrombus Dissolution and PCD Data with Different MI's for 5 μsec PD

Figure 3 depicts the returning signals from the con-focal PCD's at the 5 μsec pulse duration (PD) applications at incremental mechanical indexes (MI's). The 20 MHz PCD's detect the broadband noise emissions when bubbles undergo IC, mainly large radial oscillations and violent collapses (ANSI 2002; Holland et al. 1990, 1996). With the 5 μsec PD, these broadband noise emissions increased at 0.6 MI, with the magnitude of the emissions progressively increasing as one exceeds 0.7 MI, indicating IC became dominant at 0.7 MI. Note that %TD did not increase above control conditions until an MI of 0.7, and increased significantly at a threshold MI higher than 0.8 ($p= 0.0014$ compared to 0.2 MI). No further dissolution occurred by going beyond 0.8 MI (Figure 3B).

Thrombus Dissolution and PCD Patterns with Different MI's for 20 μsec PD

In the 20 μsec PD group, although the number of collapse signals were similar, the duration of each one of the collapse signals was four times longer than the short pulse group, so the overall root mean square of inertial cavitation was greater. Figure 4 shows %TD was significantly higher already at the 0.2 MI therapeutic impulse. Significantly higher thrombus dissolution was observed with the 20 μsec pulse duration (compared to 5 μsec pulse duration) until 0.8 MI, at which point both PDs produced equivalent thrombus dissolution. However, at higher MI's with the 20 μsec pulse duration, %TD decreased such that at the 1.4 MI, the % TD was not significantly greater than with microbubbles alone. With the 20 MHz PCD's, only low levels of IC were detected at the 0.2, 0.4, and 0.8 MI settings (left panel; Figure 4), which then increased to reach the maximum amount at the 1.2 and 1.4 MI settings.

Patterns of Thrombus Dissolution at the 20 and 5 μsec Pulse Durations

The comparison of the %TD in long versus short PD TUS is displayed in Figure 5. Already at the 0.2 MI there was significantly more thrombus dissolution for the 20 μsec pulse duration. The %TD was significantly higher in the 20 μsec therapeutic PD group until 0.8 MI, when the %TD in long and short PD group became equivalent. Percent thrombus dissolution became less as MI was increased further in the 20 μsec pulse duration, even though the magnitude of IC increased. Transmural thrombus dissolution was seen most frequently with the 0.6 and 0.8 MI settings using the 20 μsec PD (33% versus 0% 5 μsec PD at 0.6/0.8 MI; $p<0.05$), while asymmetric residual thrombus reappeared at higher MI settings with the 20 μsec PD(6/12 had asymmetric residual thrombus at the 1.2 and 1.4 MI 20 μsec treatments). Figure 6 displays the typical pattern residual thrombus seen as a function of MI when using the 20 μsec PD.

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Comparisons of Inertial Cavitation Produced with the 20 μsec pulse duration at different MI's and Different Flow Conditions

Figure 7 depicts cavitation signals from within the vessel at different flow rates as MI was increased at the 20 μsec pulse duration. The magnitude and number of IC emissions with the 20 μsec pulse duration impulses, as a function of MI transmitted through the 5cm-thick TMP, was equivalent at all flow conditions tested (a,1 ml/min; b,5 ml/min; and c,20 ml/min).

Discussion

Using an in vitro flow system, we examined the impact of alterations in therapeutic pulse duration from a diagnostic transducer in dissolving thrombi within a vessel. %TD was significantly higher with a 20 μsec PD at relatively low MI therapeutic impulses settings up to 0.6, which was before an increase in the degree of inertial cavitation was observed. At 0.8 MI, both PDs produced equivalent thrombus dissolution. As the MI was increased further with the 20 usec PD, the % TD decreased, despite higher intensity inertial cavitation emissions.

Previous studies have demonstrated with single element non-imaging transducers at 1 MHz that even without any thrombolytic drug, intermittent high MI ultrasound and intravenous microbubbles have been effective in dissolving acute thrombi (primary ultrasound fibrinolysis). Xie and colleagues investigated microbubble-mediated sonothrombolysis using a canine dialysis graft thrombus occlusion model during an intravenous lipid microbubble infusion (Xie et al. 2005). Restoration of graft flow (as an indicator of successful treatment) was seen only at MI's that induced IC. However, these were all at PD used for diagnostic imaging (<5 μsec), and thus the duration of cavitation induced by the therapeutic impulses was much shorter. The current study demonstrated that a similar degree of thrombus dissolution could be achieved with the pulse durations emanating from a diagnostic transducer. Moreover, minimal IC was required to induce primary ultrasound fibrinolysis at a slightly longer pulse duration. While higher magnitudes of IC appeared to correlate with greater thrombus dissolution at the 5 μsec pulse duration, this was not true at the 20 μsec PD, where higher magnitudes of IC were associated with less thrombus dissolution.

Previous investigators have also examined the role of both IC and SC during ultrasound-accelerated enzymatic fibrinolysis with tPA (Prokop et al. 2007). In their studies, the addition of microbubbles significantly increased lysis, but IC was present only at the start of the ultrasound exposure, while SC with low-amplitude sub-harmonic emissions persisted throughout. Datta and colleagues investigated SC and IC as possible mechanisms for enhancing thrombolysis with tissue plasminogen activator (tPA) (Datta et al. 2006). In their experiments, they mainly induced SC and were below the IC threshold, and they were able to produce consistent enhancement of thrombolysis. Since these studies all used longer PDs than those tested in this study, it is possible that their success at lower peak negative pressures was related to the longer pulse duration.

Our study shows that with slightly longer PD therapeutic impulses on a diagnostic transducer, %TD significantly increased at MI's where the magnitude of IC was small. It is

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possible that because IC is sustained at the longer PD, smaller magnitudes of IC are sufficient to achieve thrombus dissolution. Secondly, it is possible, as others have shown with tPA, that SC was more effective at thrombus dissolution at longer PD. Although we only measured the degree of IC achieved with the different MI's and pulse durations, we have shown in previous studies that both SC and IC co-exist at low to moderate MI's (Vignon et al. 2013). However, until this study, lower MI values have not been shown to be effective in dissolving thrombi without the assistance of a fibrinolytic agent. It is also possible that the longer PD resulted in bubble coalescence and translation at the lower MI (Fan et al. 2013), which may be more effective in dissolving thrombi.

Although the exact mechanism for enhanced thrombus dissolution at the lower MI cannot be completely discerned from this study, we did observe that higher magnitudes of IC at the 20 μsec PD may be associated with less thrombus dissolution. In this setting, maximal thrombus dissolution occurred at MIs of 0.6 MI, and beyond this MI the %TD tended to decrease. Conversely, the magnitude of IC, at all flow rates tested, increased slightly at 0.6 MI but dramatically increased at higher MI's. It is possible that the higher magnitude of IC induced by the higher MI impulses actually shielded the thrombus and prevented further thrombus dissolution. There is evidence for this in the visual appearance of residual thrombus after the treatment. The residual thrombus formation tended to be asymmetric at the higher MI's, as would be expected if the microbubbles functioned as "perfect reflectors" at this higher magnitude of IC. This shielding effect has been described by others using 1 MHz therapeutic impulses and long PD at higher MI settings (Ammi et al. 2012). Although this may be a potential explanation for the decreasing thrombus dissolution as MI (and degree of IC) are increased at the 20 μsec PD, such a shielding effect has not been observed in microvascular thrombi models (Leeman et al. 2012). In this setting, microbubbles are much more dispersed and thus would not be expected to travel in channels, but rather as single entities within a capillary and arteriolar matrix of thrombi.

Other factors play a role in ultrasound induced thrombus dissolution, including clot vibration from acoustic radiation force (Chen et al. 2014). In this study, we believe the effect of radiation force was minor at the 50 Hertz frame rate used for therapeutic applications, but our ability to discern the impact of this effect during IC conditions will be improved with ultra-high speed microscopy imaging.

Clinically, such findings indicate that software modifications in diagnostic imaging transducers can result in significant therapeutic effects at mechanical indices that are well within any Food and Drug Administration limits. Our study findings also indicate that an applied mechanical index can be too high to achieve therapeutic effects with longer pulse durations, and thus feedback on the intensity of cavitation induced may be required. More importantly, thrombus dissolution with diagnostic ultrasound and microbubbles can be achieved without any fibrinolytic therapy, and thus eliminate the risk that lytic therapy imparts in the setting of stroke or acute myocardial infarction. Although diagnostic imaging frequencies are limited on how low a frequency they can transmit, we chose the lowest one possible in order to lower the threshold for inducing cavitation. Diagnostic ultrasound also allows for low MI imaging in order to visualize when microbubbles are present within the thrombus, and thus guide when to apply the therapeutic impulses. However, this

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visualization of microbubbles within the thrombus may only be possible with earlier aged thrombi that have not undergone clot retraction (Sutton et al. 2013). Further work is needed to determine what effect these histologic variables in thrombus composition and structure have on ultrasound and microbubble induced thrombus dissolution without fibrinolytic therapy.

In conclusion, our study demonstrates that longer PD impulses emanating from a diagnostic transducer at lower peak negative pressures can effectively recanalize thrombosed vessels. Unlike shorter PDs, the magnitude of IC induced does not correlate with the amount of thrombus dissolved. Rather, there is a reduction in thrombus dissolved at higher MI's, which may be due to a shielding of the thrombus by the microbubbles undergoing cavitation. Since these observations were done with a tissue mimicking phantom, they may have relevance in choosing the optimal settings for primary ultrasound thrombolysis in acute coronary thrombosis.

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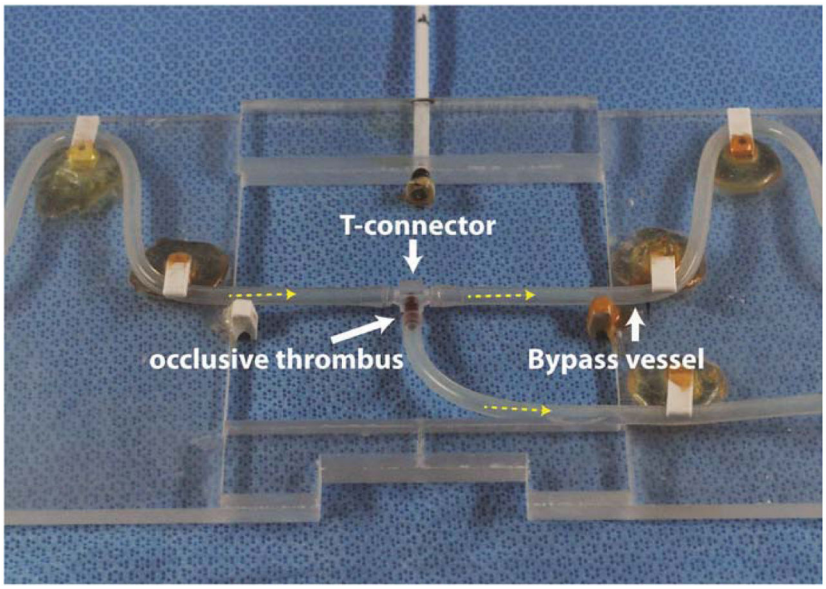

Figure 1.
T connector with occlusive thrombus and bypass vessel. The yellow arrows indicate the direction of flow.

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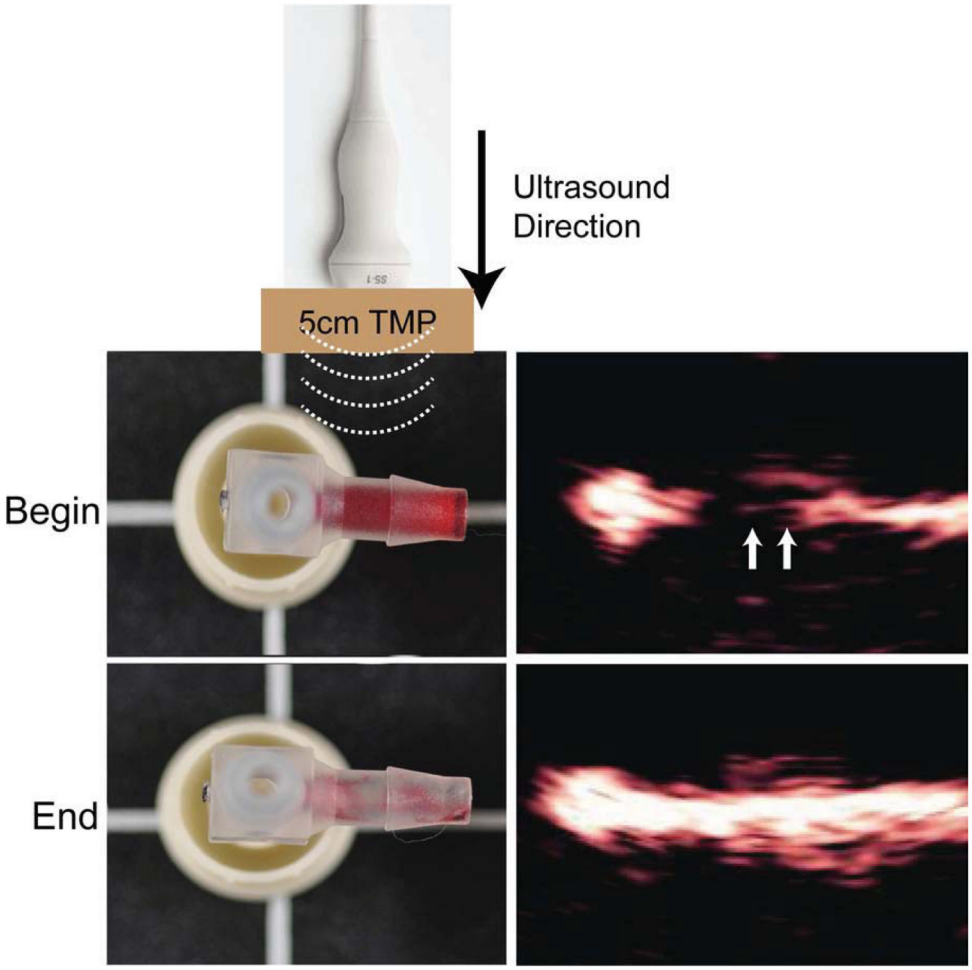

Figure 2.

Low MI Imaging prior to therapeutic impulses (top panels) demonstrate a small thin channel of microbubbles within the thrombus (white arrow) despite a visually occlusive thrombus on the left. Following treatment with the 0.6 MI therapeutic impulses at 20 μsec pulse duration, there is transmural clearance of the thrombus (lower panels).

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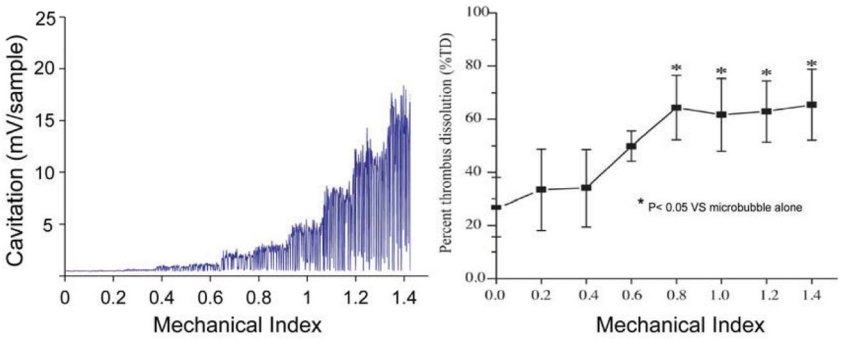

Figure 3.

Inertial cavitation emissions and % thrombus dissolution from the 5 μsec pulse durations on the modified diagnostic ultrasound transducer. Note that as mechanical index was increased to beyond 0.7, there was a threshold increase in magnitude and number of cavitation emissions, which correlated closely with thrombus dissolution.

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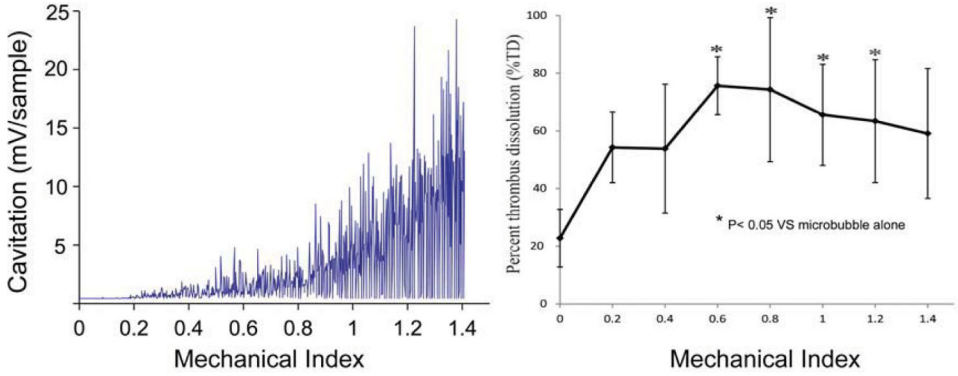

Figure 4.

Inertial cavitation emissions from the 20 μsec pulse durations on the modified diagnostic ultrasound transducer. Unlike with the 5 μsec pulse duration, here there was thrombus dissolution occurring already at 0.2 MI which peaked at 0.6 MI. This occurs before we see any significant increase in the magnitude of inertial cavitation emission impulses.

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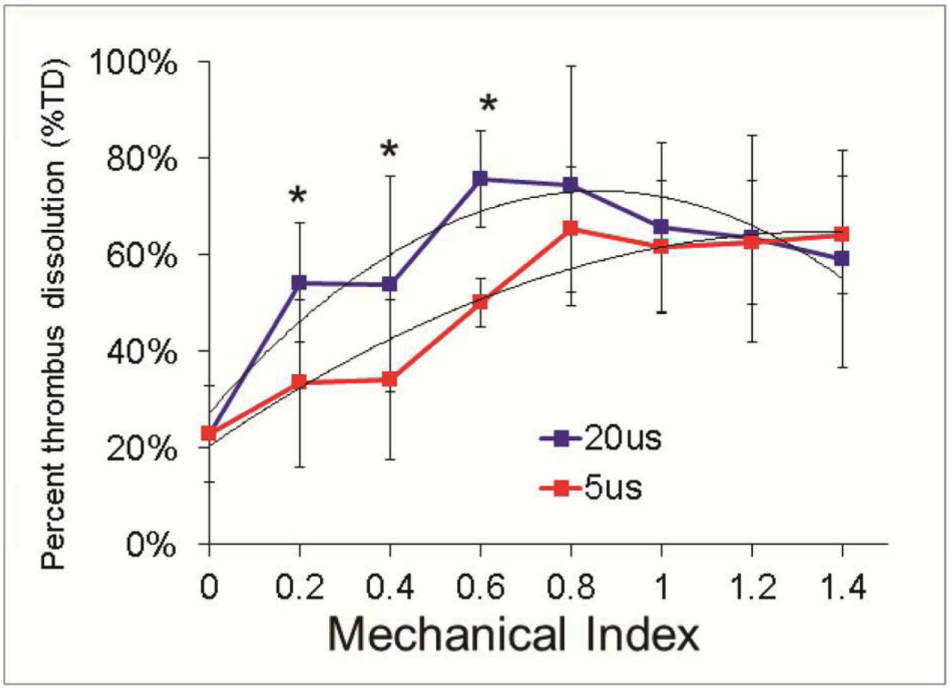

Figure 5.

The increased thrombus dissolution within a 4mm vessel observed with a longer pulse duration (20 μsec) when compared to a shorter pulse duration at the same MI. Not until an MI of 0.8 is the degree of thrombus dissolution equivalent. *$p<0.05$ compared to a 5 μsec pulse duration therapeutic impulse at the same MI.

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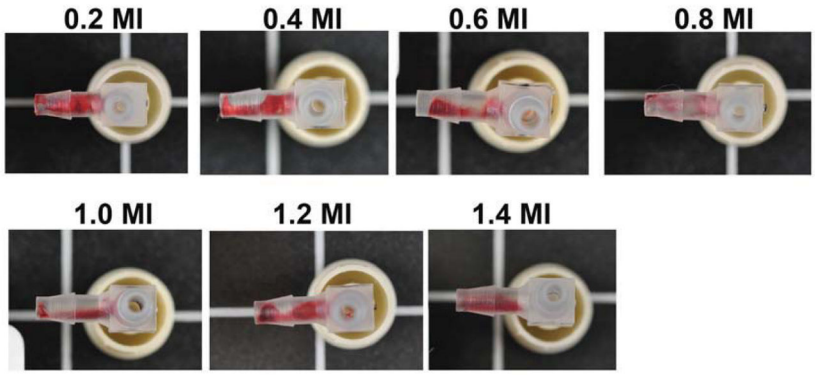

Figure 6.

Examples of residual thrombus present following 20 μsec PD therapeutic impulses as MI was increased. Note at 0.8 MI, there is transmural clearance of thrombus.

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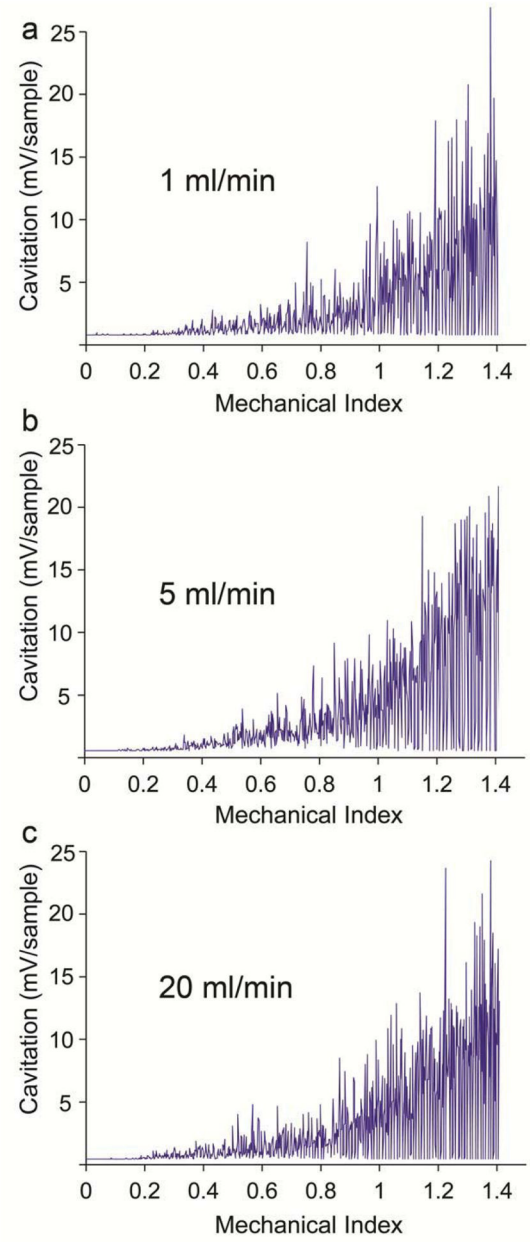

Figure 7.

PCD derived inertial cavitation emissions from within the flow system at different flow conditions (a,1 ml/min; b,5 ml/min; and c,20 ml/min). There was no difference in the magnitude or number of emissions at the different flow conditions.