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● *Original Contribution*

# ACTIVATION, AGGREGATION AND ADHESION OF PLATELETS EXPOSED TO HIGH-INTENSITY FOCUSED ULTRASOUND

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**Abstract—Using platelet-rich plasma, we investigated the effect of 1.1-MHz continuous wave high-intensity focused ultrasound (HIFU) on platelet activation, aggregation and adhesion to a collagen-coated surface. Platelets were exposed for durations of 10–500 s at spatial average intensities of up to 4860 W/cm$^2$. To avoid heating effects, the average temperature in the HIFU tank was maintained at 33.8 ± 4.0°C during platelet experiments. Flow cytometry, laser aggregometry, environmental scanning electron microscopy and passive cavitation detection were used to observe and to quantify platelet activation, aggregation, adhesion to a collagen-coated surface and associated cavitation. It was determined that HIFU can activate platelets, stimulate them to aggregate and promote their adherence to a collagen-coated surface. In principle, HIFU can stimulate primary, or platelet-related, hemostasis. Cavitation was monitored by a passive cavitation detector during aggregation trials and was quantified to provide a relative measure of the amount of cavitation that occurred in each aggregation trial. Regression analysis shows a weak correlation ($r^2 = 0.11$) between aggregation and ultrasound intensity, but a substantial correlation ($r^2 = 0.76$) between aggregation and cavitation occurrence. (E-mail: poliachi@u.washington.edu)** 

*Key Words:* **Ultrasound, Cavitation, Platelets, Aggregation, Bioeffects, HIFU.**

## INTRODUCTION

The ability to perform acoustic hemostasis using high-intensity focused ultrasound (HIFU) has been investigated by a number of researchers (Delon-Martin et al. 1995; Hynynen et al. 1996a, 1996b; Martin et al. 1999; Vaezy et al. 1997, 1998, 1999a, 1999b). In the acoustic hemostasis studies performed by Vaezy et al. (1997, 1998, 1999a, 1999b), the dominant mechanism seems to be a temperature rise sufficient to cauterize the tissue at the bleeding site. Acoustic cavitation, which is related to the expansion and compression of gas bubbles caused by the applied acoustic field, was not monitored during these HIFU exposures. Delon-Martin et al. (1995) attributed the formation of venous thromboses in normal blood vessels to thermal damage of the vessel. Using a passive cavitation detector, they did not detect any cavitation activity during 7.31-MHz HIFU exposures at spatial average temporal peak intensities of 167 W/cm$^2$. Hynynen et al. (1996b) suggested that a mechanical/thermal stimulus ceased blood flow in a healthy vessel after application of HIFU. They purposely applied HIFU above the cavitation threshold (peak intensity 6500 W/cm$^2$), verified by strong subharmonic noise in the spectrum of acoustic emissions monitored by a passive cavitation hydrophone, to constrict the vessel. Without the cooling blood flow, sonications at lower intensities (peak intensity 2800 W/cm$^2$) were used to produce thermal coagulation of the vessel. Our studies concentrate on the stimulation of platelet-related hemostasis under controlled temperatures such that other effects of HIFU can be evaluated.

If a vessel is damaged, subendothelial proteins such as collagen are exposed. The hemostatic process starts when platelets come into contact with collagen. Platelets adhere to the exposed collagen in the damaged vessel, become activated, spread out and release their contents, thereby recruiting other platelets, which leads to the formation of a primary hemostatic plug. Platelet adherence and activation in turn lead to activation of the

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coagulation system resulting in formation of fibrin that stabilizes the hemostatic plug.

Platelet activation may be stimulated by exposure to collagen, epinephrine, adenosine diphosphate (ADP), thrombin, platelet-activating factor (PAF) or strong shearing forces. Once activated, platelets release a variety of factors that stimulate other platelets. Thermal effects can also increase platelet activity. If the temperature of a platelet rises to a point where the platelet membrane becomes disorganized, platelet contents may leak out and cause activation of other platelets.

Prior studies of the effect of ultrasound on platelets have shown that unfocused ultrasound at therapeutic intensities (0.2–16 W/cm$^2$) can decrease the time required to form a clot after addition of calcium to an *in vitro* sample (Williams et al. 1976b), and create platelet aggregates in the microcirculation of subcutaneous tissues of guinea pig ear (Zarod and Williams 1977). Unfocused therapeutic ultrasound can also stimulate the release of the platelet-specific protein $\beta$-thromboglobulin (Williams et al. 1978), change platelet morphology and function *in vitro* (Williams et al. 1976a), increase platelet calcium concentrations and stimulate platelet aggregation *in vitro* (Samal et al. 2000). In platelet samples supplemented with microbubble contrast agent, platelet destruction occurred for focused 1-MHz pulsed ultrasound exposures of 730 W/cm$^2$ ($I_{SPPA}$), corresponding to 3.5 MPa peak negative pressure (Everbach et al. 1998). With unfocused ultrasound, Chater and Williams (1977) reported that platelet aggregation was more likely to occur at lower ultrasound frequencies, with exposures at spatial average intensities of 3.75 W/cm$^2$ causing more aggregation at 0.75 MHz than at 1.5 or 3.0 MHz. Although cavitation was not measured, Chater and Williams (1977) suggested the mechanism that induced aggregation was cavitation. In a series of *in vitro* experiments, we have investigated the ability of HIFU to activate platelets, stimulate them to aggregate and cause them to adhere to a collagen-coated surface that simulates a wound. Our results suggest that, at physiologic temperatures, cavitation plays a role in enhancing platelet activity.

## MATERIALS AND METHODS

Studies on human subjects were carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants, and the study was approved by the University of Washington Human Subjects Review Committee. Immediately before each experiment, blood was drawn from healthy donors and anticoagulated by addition of nine parts blood with one part 0.105 mol/l sodium citrate. Blood handling was minimized and blood was typically used within 2 h of being drawn, while no experiment lasted longer than 3 h. Platelet-rich plasma (PRP) was used for the experiments. Platelet-rich plasma was prepared by centrifuging whole blood at 800 rpm for 10 min and removing the PRP supernatant. Based on automated cell counting, the PRP contained approximately 200,000 platelets per microliter plasma. Platelet-poor plasma (PPP), used to calibrate the aggregometer, was prepared by centrifuging whole blood at 2500 rpm for 10 min to remove the platelets and other cellular components from the plasma. After ultrasound exposure in activation studies, PRP samples were placed into polypropylene microcentrifuge tubes (CMS, Inc., Federal Way, WA, USA) and analyzed in a flow cytometer within 30 min as described below.

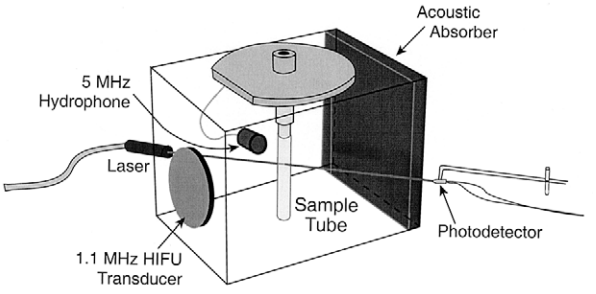


Fig. 1. Experimental arrangement for high-intensity focused ultrasound exposures for platelet activation and aggregation studies. The 1.1-MHz transducer is powered by a function generator applied to an amplifier, with the signal routed through a matching network prior to exciting the transducer. A 5-MHz hydrophone acts as a passive cavitation detector and is aligned confocally with the 1.1-MHz transducer. An acoustically transparent sample chamber is stationed so that the focus of the 1.1-MHz transducer is located within the sample volume. During aggregation studies, a laser is aimed through the sample chamber, and a photosensor detects the amount of light transmitted through the sample. The photosensor output is connected to an oscilloscope. Hydrophone data from exposures are peak-detected before analysis. LabVIEW programs are utilized for final signal processing. For platelet adhesion studies, a rectangular sample chamber is used, with its long axis parallel to the axial direction of the 1.1-MHz transducer.

All ultrasound exposures occurred in the HIFU system that has been described in detail elsewhere (Poliachik et al. 1999). Figure 1 shows the experimental arrangement. Briefly, in an acrylic tank containing degassed phosphate-buffered saline (PBS), an acoustic absorber was placed in the tank opposite the insonating 1.1-MHz transducer (Sonic Concepts, Woodinville, WA, USA). A 5-MHz focused hydrophone (Sonic Concepts, Woodinville, WA, USA) with a bandwidth of 2 MHz was positioned in the wall of the tank, 90° from the insonating transducer. The tank was constructed specifically to allow the transducer to be mounted in the sidewalls, permitting both the HIFU transducer and the hydrophone to be aligned confocally within the tank.

Table 1. Equivalent measured pressures and measured intensities for HIFU transducer

| Acoustic intensity ($W/cm^2$) | Positive acoustic pressure (MPa) | Negative acoustic pressure (MPa) |
|---|---|---|
| 500 | 2.67 | 2.10 |
| 1000 | 3.79 | 2.64 |
| 1500 | 4.66 | 2.95 |

The 1.1-MHz HIFU transducer was mounted in one end of the acrylic tank. The focal zone of the 1.1-MHz transducer was 1.5 mm × 8.2 mm, based on the full width at half maximum pressure points. The cylindrical sample chamber was centered on the geometric focus of the source, and was held by a positioning block in the bottom of the tank. For stirred samples in aggregation experiments, a magnetic stir bar was placed in the sample chamber, with the stir plate (Scinics Co., Ltd., Tokyo, Japan) situated beneath the HIFU tank. Rectangular sample chambers used in adhesion studies were positioned within the tank using a three-axis motion stage (Techno Isel, New Hyde Park, NY, USA). The sample chambers were positioned in the tank so that the HIFU focus was situated in the center of the rectangular sample chamber.

The transducer was energized by a function generator (HP33120A, Hewlett Packard, Palo Alto, CA, USA) applied to a power amplifier (AP-400B, ENI, Rochester, NY, USA) and matching network (Sonic Concepts, Woodinville, WA, USA). The function generator was used to produce the 1.1-MHz frequency and the voltage of the input signal. Immediate application of high intensities had a tendency to pit and scar the acoustically transparent sample chambers at the beam focus, thereby causing partial reflections of the ultrasound energy. To avoid damage to the sample chamber when the power was initially activated, a LabVIEW (National Instruments Corporation, Austin, TX, USA) program was written to increase the voltage applied to the transducer over 5–10 s. All ultrasound exposures were monitored by a CCD camera (Sony Electronics, Inc., Park Ridge, NJ, USA) and the output recorded using a VCR (Samsung Electronics Co., Ltd., Seoul, Korea).

The HIFU transducer used in these experiments was calibrated using a radiation force balance as in Poliachik et al. (1999). Exposure conditions are reported in terms of spatial average intensity. Comparison measurements were acquired using a calibrated 0.6-mm diameter PVDF TNU001A needle hydrophone (NTR Systems, Inc., Seattle, WA, USA) in combination with a three-way positioner and an oscilloscope (LeCroy LT344, Chestnut Ridge, NY, USA). At higher pressures, nonlinearity increased in the system, thus producing a signal in which the positive pressure was greater than the negative pressure. Table 1 lists equivalent measured spatial average intensities and measured pressures for the HIFU transducer.

The temperature within the HIFU tank was measured using a 0.013-cm diameter Type K thermocouple (Omega Engineering, Inc., Stamford, CT, USA) connected to a 52 K/J thermometer (Fluke Corporation, Everett, WA, USA). The starting temperature of the tank was 30°C. If a temperature rise occurred during HIFU exposure, cool degassed PBS was pumped into the tank after each exposure until the temperature was 30 ± 1°C. Pulsing schemes were employed to maintain a temperature below 42°C during adhesion experiments, which used sample chambers that trapped heat more effectively than the cylindrical sample chambers. This particular temperature control scheme was used because the response time of automatic temperature control was not adequate. The average temperature in the tank during the experiments was 33.8 ± 4.0°C.

During ultrasound exposure, data from the hydrophone were collected using an oscilloscope (LeCroy 9301A, Chestnut Ridge, NY, USA) and a LabVIEW program. The signal from the 5-MHz hydrophone was routed through a peak detector circuit (PKD01, Analog Devices, Cambridge, MA, USA) and then into the oscilloscope to capture hydrophone data for the entire exposure time at the maximum available sample rate of 500 samples per second. The LabVIEW program triggered the transducer and oscilloscope and saved the data to disk on a pentium computer (Gateway, North Sioux City, SD, USA).

Data collected from the hydrophone during the experiment were analyzed to determine whether cavitation had occurred. A LabVIEW program calculated the integral of the demodulated data that were above a baseline noise level for each trial, as used previously (Poliachik et al. 1999). Because of the low sample rate, the program calculates an area under the curve that provides an estimate of the amount of cavitation occurring during a trial. We term this quantity the relative cavitation dose (RCD). The data were analyzed to allow a comparison of the relative amount of cavitation occurring in each trial.

Cylindrical sample chambers for the PRP specimens in activation studies consisted of a delrin plug and tube connected by acoustically transparent biocompatible polyester tubing, as used previously (Poliachik et al., 1999). The exposure volume was 1 ml. The top surface of the sample was exposed to air, so a sample size of 1.3 ml was used to move the air interface away from the transducer focus. Platelet activation samples were exposed to continuous wave (CW) HIFU intensities of 0–2200 $W/cm^2$. Because aggregation is an undesirable effect during activation studies (during aggregation, the binding sites used in the activation assays are occupied),

exposure times for PRP samples were limited to 100 s to avoid platelet aggregation.

Platelet activation was measured using flow cytometry. When a platelet is activated, granules are released and the protein P-selectin is expressed on the surface of the platelet. Also, phospholipids that make up the platelet membrane invert such that anionic phospholipids are exposed. These anionic phospholipids are essential for activation of the coagulation cascade. As described by Tait et al. (1999), fluorescently labeled antibodies for the P-selectin protein (phycoerythrin-anti-CD62P, Becton-Dickinson, Franklin Lakes, NJ, USA) and labeled annexin V (Wood et al. 1996) for anionic phospholipids were added to PRP samples that had been exposed or sham-exposed to HIFU. Samples were then analyzed in a flow cytometer (XL-MCL, Beckman Coulter, Fullerton, CA, USA). The intensity of fluorescence per platelet was measured. The background fluorescence of platelets was determined by adding fluorescently labeled nonspecific IgG. Platelets with fluorescence above the background level were defined as activated platelets. The percentage of activated platelets was determined before and after exposure to HIFU.

Aggregation was measured within the cylindrical sample chambers, as used in activation studies, with a laser and a photodetector system. A 670-nm laser (Quarton Inc., Taiwan) was mounted outside the acrylic tank and positioned to locate the beam within the sample chamber with the emerging beam striking the OP803SL photosensor (Newark Electronics, Kirkland, WA, USA), also located outside the tank. The signal from the photosensor was recorded on a LeCroy oscilloscope. CW HIFU intensities of up to 2300 W/cm$^2$ were used in the 500 s HIFU exposures. During the exposures, data from the photosensor and the cavitation data from the hydrophone were collected on an oscilloscope for the duration of exposure.

To investigate the ability of a limited amount of HIFU-induced cavitation to aggregate platelets, PRP in the cylindrical sample chambers was exposed to CW HIFU for 10 s and then stirred for 490 s with a magnetic stir bar. Intensities of up to 4860 W/cm$^2$ were used. Photosensor and cavitation data were collected for the combined 10 s HIFU exposure and 490 s stirring time.

Glass slides were coated with collagen by adding 10 $\mu$l of a collagen-containing solution (No. 385 collagen, Chrono-PAR, Chrono-Log, Haverton, PA, USA), and then drying at room temperature for 12 h. For platelet adhesion studies, the sample chambers consisted of a 1-cm square polystyrene cuvette (VWR, San Francisco, CA, USA) cut to 3 cm in length. The sample chamber was positioned with the long axis parallel to the axis of the 1.1-MHz transducer, as depicted in Fig. 2a. A collagen-coated glass slide was affixed with silicone adhesive on one end, whereas parafilm was sealed over the transducer-facing end after loading the chamber with PRP. Exposure times were 120 s CW for all trials, at intensities of up to 2120 W/cm$^2$. When pulsed ultrasound was used to prevent sample heating, the total exposure time (HIFU on-time) was maintained at 120 s.

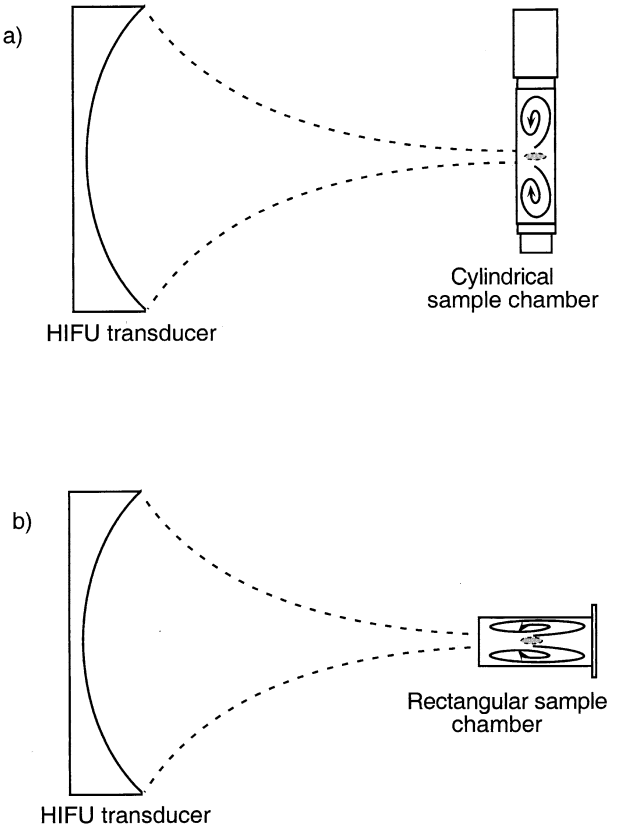


Fig. 2. Schematic of flow patterns in the sample chambers induced during CW HIFU exposures. For both types of sample chambers, the 1.1-MHz HIFU transducer focus is positioned in the center of the sample chamber. The general flow pattern is shown in (a) the rectangular sample chamber used in platelet adhesion studies, and (b) the cylindrical sample chamber used in activation and aggregation studies.

After HIFU exposure, the collagen-coated glass slide was removed from the cuvette and the slide was gently rinsed with PBS. Platelets were fixed using Wright's stain. Scanning electron micrographs of the collagen-coated surface were obtained using an environmental scanning electron microscope (FEI Co., Hillsboro, OR, USA) that operated in a vacuum of 5 to 15 Torr.

The correlation coefficient was calculated using regression analysis. Statistica/Mac™ software (StatSoft™, Tulsa, OK, USA) was used for all statistical calculations.

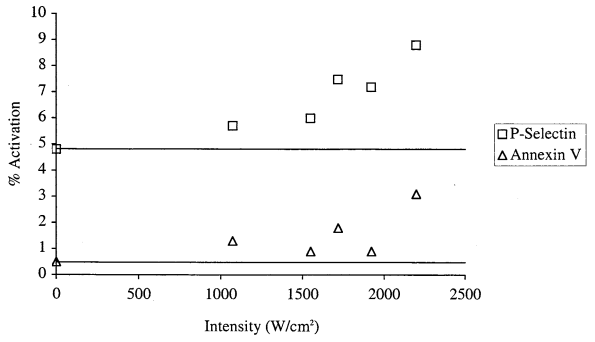


Fig. 3. Platelet activation response to HIFU intensities. Flow cytometry was used to measure expression of P selectin (□) and anionic phospholipids, as marked with annexin V (△). Baseline values of annexin V and P-selectin are noted by horizontal lines. Platelet activation tends to increase as intensity increases.

## RESULTS

PRP samples in cylindrical sample chambers were exposed to HIFU CW intensities ranging up to 2200 W/cm$^2$ for 100 s. Exposure of PRP to HIFU resulted in stirring of the platelets within the chamber due to acoustic streaming and radiation pressure. Figure 2b shows a schematic of the flow pattern of the platelets within the cylindrical sample chamber. Based on the size of the HIFU focus and a 1-ml sample exposure volume, only about 2% of the sample was within the beam focus during exposure, but vigorous HIFU-induced stirring within the sample chamber allowed an estimated 85% of the sample to be exposed to HIFU. Exposure of PRP to HIFU showed a trend of intensity-dependent increase in platelet activation markers present on the platelet surface (Fig. 3). Without HIFU exposure, 4.8% of platelets expressed P-selectin on their surface (a measure of platelet granule release), while 0.5% bound Annexin V (a measure of anionic phospholipid exposure). After 100 s of 2200 W/cm$^2$ HIFU exposure, 8.8% of platelets were expressing P-selectin and 3.1% bound Annexin V, indicating that HIFU had stimulated both granule release and anionic phospholipid exposure.

Because HIFU exposures of 100 s successfully produced platelet activation, platelet aggregation, which cannot occur without platelet activation, was used to quantify the effect of HIFU exposure on platelet activity. Platelet-rich plasma samples in cylindrical sample chambers were exposed to HIFU CW intensities of up to 2300 W/cm$^2$ for 500 s. Exposure of PRP to HIFU resulted in platelet aggregation in some cases. Figure 4 shows the

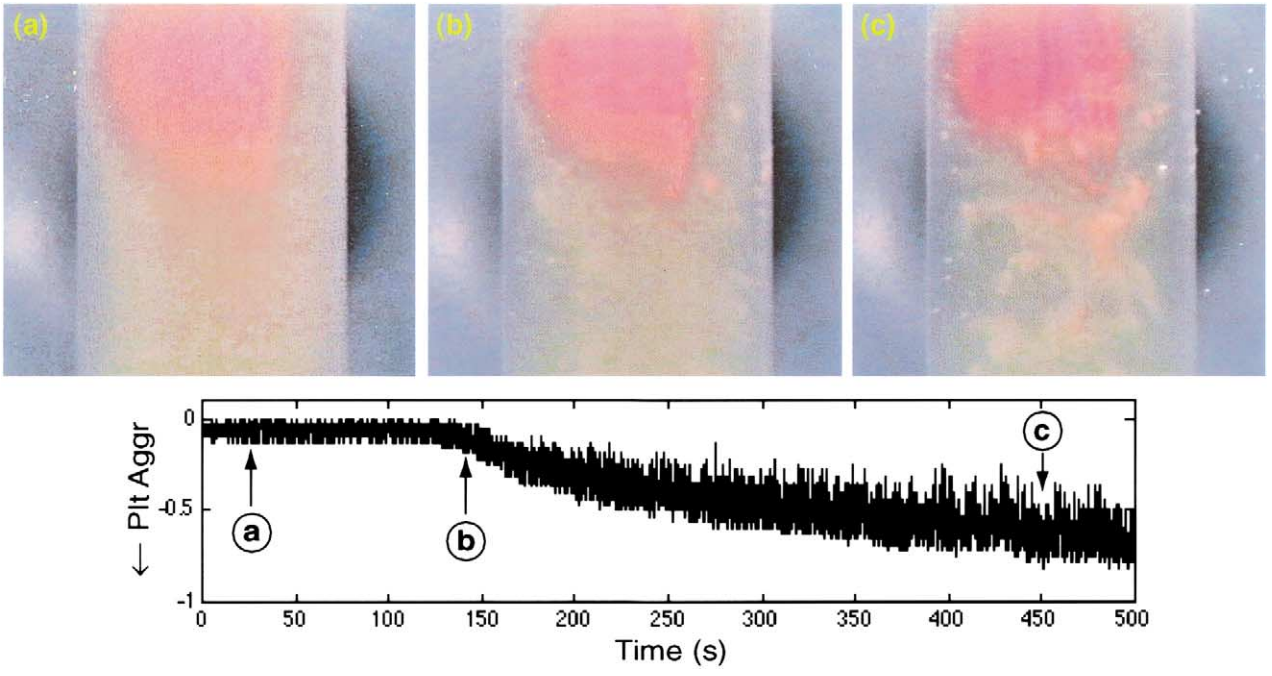


Fig. 4. Video frames showing progression of aggregation. (a) Depicts platelet-rich plasma, which is cloudy and thus does not transmit light, as shown on the associated aggregation curve by arrow “a”. (b) Depicts small aggregates formed in the sample chamber, allowing some light transmittance as shown by the decreasing voltage noted by arrow “b” on the aggregation curve. (c) Depicts nearly full aggregation and greater light transmittance, as shown by arrow “c” on the aggregation curve. The red spot on tube is the location of laser beam.

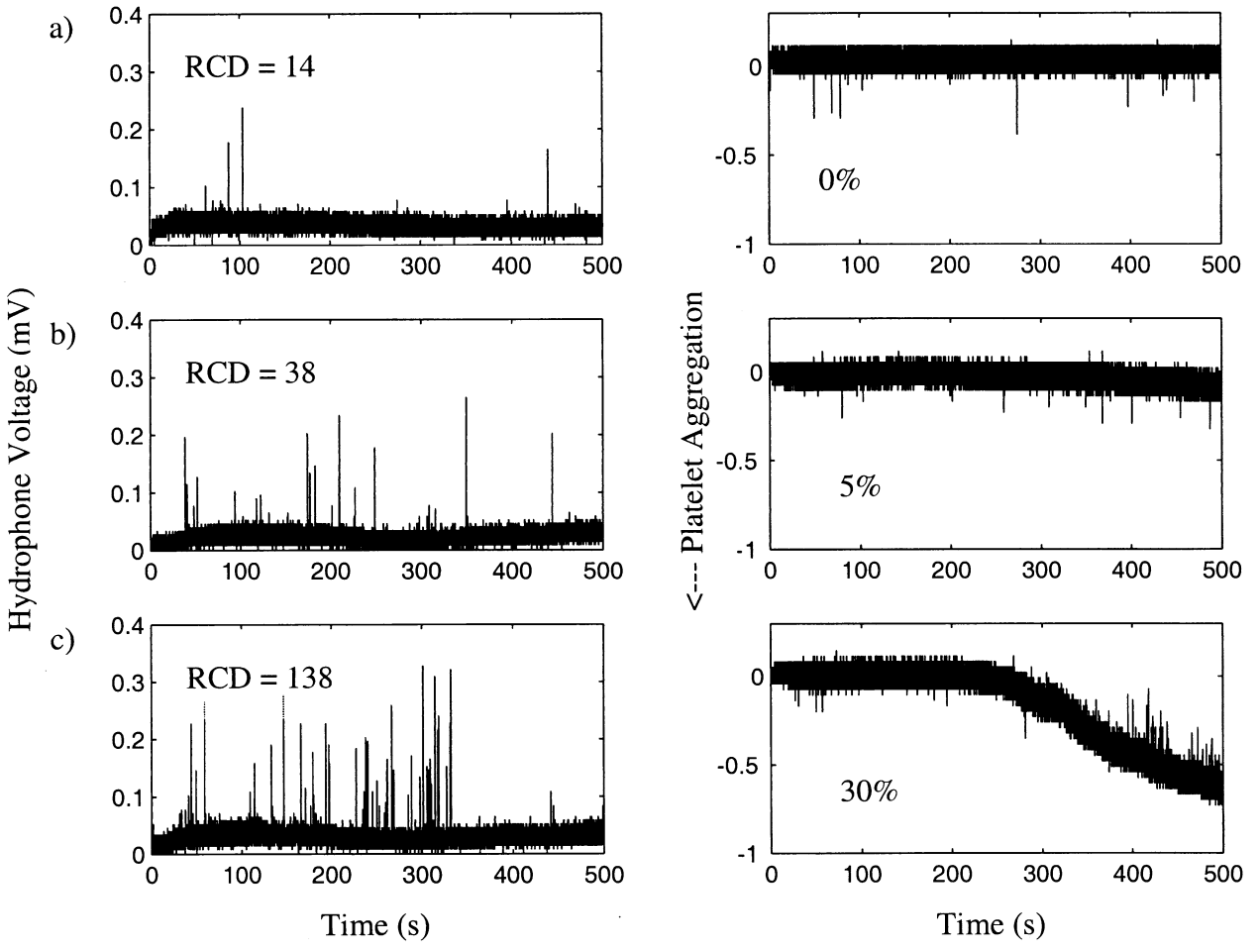


Fig. 5. Cavitation and associated aggregation. These data demonstrate the amount of cavitation that occurred during a trial (left column) and its associated amount of aggregation (right column). (a) For exposure at 1044 W/cm$^2$, the amount of cavitation was measured at a relative cavitation dose (RCD) of 14 and the exposure produced 0% aggregation. (b) At 1535 W/cm$^2$, RCD increased to 38 and aggregation increased to 5%. (c) The 2180 W/cm$^2$ exposure produced an RCD of 138 and aggregation of 30%, thus suggesting that a higher cavitation dose leads to greater aggregation.

time course of visual changes and platelet aggregation in the PRP during exposure to HIFU. Before HIFU exposure, the PRP was cloudy due to the suspended platelets (Fig. 4a). After initiating HIFU exposure, the platelets began streaming around the HIFU focus, as in Fig. 2. The first evidence of platelet activation on video microscopy was the appearance of microscopic aggregates in the sample, which appeared before any changes in laser aggregometry. When the aggregometry began to show a change from baseline, visible clumps of platelets were seen streaming within the sample chamber (Fig. 4b). As aggregation continued, the platelet clumps became larger and the background plasma became clear (Fig. 4c). A sample was considered to be aggregated fully when the photodetector voltage matched the voltage measured when a sample of PPP was placed in the sample chamber. Full aggregation was not measured in any sample, possibly due to excessive cavitation, which may rip platelets apart such that they are no longer capable of aggregation, yet still scatter light from the laser. Quantification of platelet destruction resulting from HIFU exposure was not attempted because platelet counts are not possible once the platelets aggregate.

Figure 5 shows representative cavitation data (left column) collected during aggregation trials along with associated aggregation curves (right column). In the aggregation curves, as platelet aggregation increases, photodetector voltage decreases. Figure 5a depicts data from a HIFU exposure at an intensity of 1045 W/cm$^2$, which resulted in little cavitation (RCD = 14) and no detectable aggregation (0%). A higher intensity of 1535 W/cm$^2$ was used in Fig. 5b, resulting in higher levels of cavitation (RCD = 38) and the start of aggregation (5%) by 500 s. Finally, 2180 W/cm$^2$ was used in Fig. 5c, resulting in a

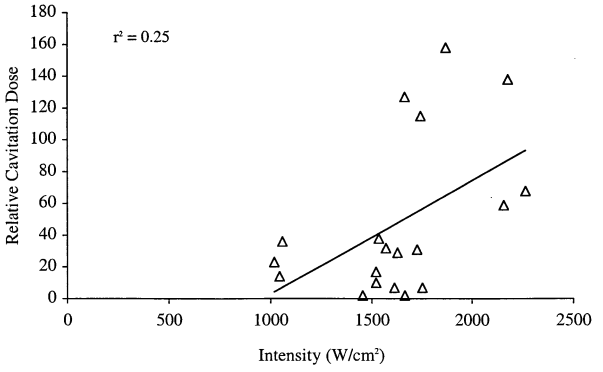


Fig. 6. Correlation between the HIFU intensity and RCD occurring in samples of PRP exposed to CW HIFU for 500 s. A correlation coefficient of $r^2 = 0.25$ demonstrates that HIFU intensity and cavitation occurrence are not strongly related.

still higher level of cavitation (RCD = 138), with aggregation starting near 250 s and reaching a maximum aggregation of 30% at 500 s.

Using various intensities, aggregation of platelets and associated cavitation were measured during each trial. Figure 6 shows cavitation measured as a function of HIFU intensity. Although cavitation tended to increase as intensity was increased, the correlation between intensity and cavitation was only $r^2 = 0.25$. Figure 7 shows percent aggregation both as a function of HIFU intensity ($r^2 = 0.11$) and of measured cavitation ($r^2 = 0.76$). In Fig. 7, as the relative cavitation dose increases, so does aggregation. It is seen that aggregation correlated better with cavitation than with intensity. This is easily understood because cavitation is somewhat of a stochastic process, related to the number and composition of nucleation sites available in each sample.

To investigate the possibility that HIFU-induced cavitation initially rips a few platelets apart, thus releasing their contents such that other platelets may be activated, short and intense HIFU exposures followed by stirring with a magnetic stir bar were conducted at various intensities. High-intensity focused ultrasound exposures of 10 s followed by stirring with a magnetic stir bar were conducted at various intensities. Control samples that were not exposed to HIFU were stirred for 500 s, and resulted in no detectable aggregation. Figure 8 shows examples of results from a 10 s exposure followed by stirring. In Fig. 8a, an intensity of 3630 W/cm$^2$ resulted in an RCD of 7 with no detectable aggregation. In Fig. 8b, an intensity of 4055 W/cm$^2$ produced an RCD of 182 with an aggregation of 12.6% after 490 s of stirring. In Fig. 8c, an intensity of 4860 W/cm$^2$ produced an RCD of 2845 and an aggregation of 25.2% after stirring. Thus, sufficient cavitation during brief HIFU exposure seems to lead to measurable aggregation in samples of PRP.

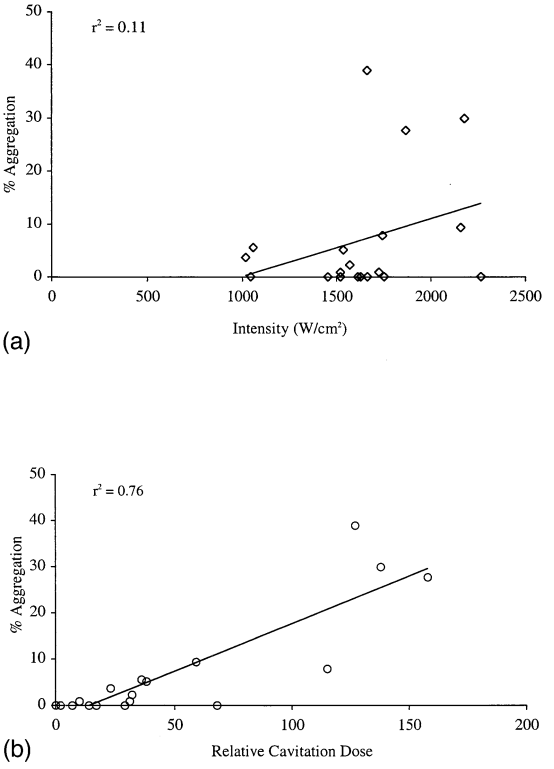


Fig. 7. (a) The correlation between the HIFU intensity used in exposures of PRP samples and the associated aggregation. Aggregation increases with an increase in intensity, with $r^2 = 0.11$, demonstrating that the two are not strongly related. (b) The relationship between the amount of cavitation and the associated aggregation. As RCD increases, so does the amount of aggregation, with substantial corelation coefficient of $r^2 = 0.76$.

Although platelet activation and aggregation are important steps in platelet-related hemostasis, the platelet clump must adhere to a surface to provide a solid base upon which a full thrombus may grow. In the body, platelets adhere to subendothelial proteins such as collagen. To demonstrate that HIFU exposure can stimulate platelets to adhere to collagen, we simulated a wound by using collagen-coated surfaces that were exposed to PRP (sham) and PRP plus HIFU. Figure 9 shows environmental scanning electron microscope (ESEM) images of a control showing a collagen-coated surface in which no HIFU or PRP was applied, a sham collagen-coated surface that had been exposed to PRP for 5 min, and a sample with platelets attached to a collagen-coated surface resulting from 2 min of HIFU exposure at 1000 W/cm$^2$. Visible in each figure are collagen strands, whereas the HIFU-exposed sample also contains clumps of platelets attached to the collagen strands. Similarly, HIFU exposures from 960 to 2120 W/cm$^2$ for 2 min caused platelets to adhere to collagen (not shown).

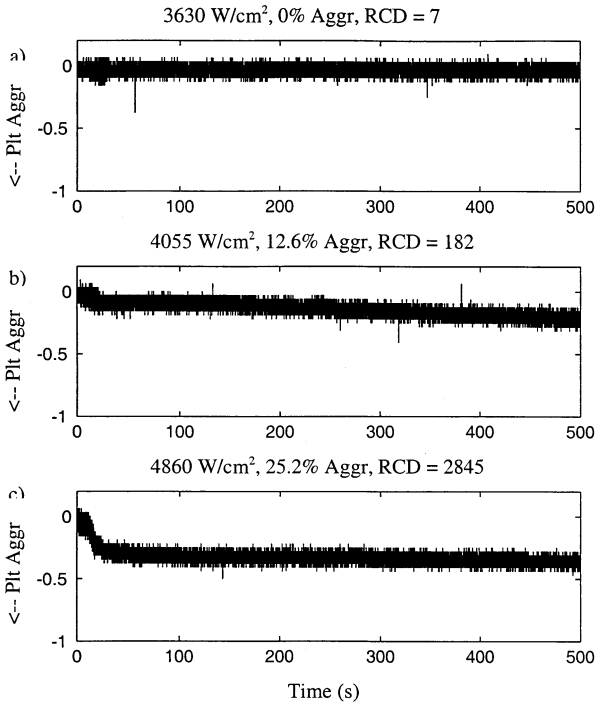


Fig. 8. Samples of PRP were exposed to 10 s of HIFU followed by stirring with a stir bar for 490 s to determine whether initial HIFU-induced cavitation was sufficient to disrupt a few platelets, whose contents are then released enabling activation of other platelets. (a) Very little cavitation occurred (RCD = 7) and no measurable aggregation occurred. (b) An RCD of 182 indicates greater cavitation activity and a resulting aggregation of 12.6%. (c) Data from a trial with extensive cavitation (RCD = 2845), and a resulting aggregation of 25.2%, thereby suggesting that an initial burst of cavitation is sufficient to release platelet contents, which can then activate other platelets.

## DISCUSSION

Samples of PRP were exposed to HIFU and analyzed to determine the amount of platelet activation, aggregation and adhesion to a collagen-coated surface. Platelet-rich plasma exposed to HIFU expressed P-selectin and anionic phospholipids, with expression increasing with acoustic intensity. Thus, HIFU can stimulate the platelet release reaction and platelet membrane reorganization. Anionic phospholipid exposure is a requirement for activation of the coagulation cascade. Part of the hemostatic effect attributed to HIFU may be due to platelet activation and acceleration of coagulation. Aggregation studies did not reveal a significant aggregation threshold based on intensity, although aggregation did generally increase with intensity. Measurements of cavitation during aggregation trials show a good correlation between cavitation and aggregation. A higher cavitation dose produced a higher degree of platelet aggregation. Platelet adhesion trials demonstrated that HIFU exposure of PRP near a collagen-coated surface for intensities between 960 and 2120 W/cm$^2$ for 2 min can cause platelets to adhere to the collagen, whereas shams do not show platelet adhesion. This result indicates that HIFU may prove to be an effective means to speed up platelet plug formation in low flow systems without causing collateral damage to surrounding tissues as might be the case with HIFU cauterization.

HIFU exposures of PRP above 1750 W/cm$^2$ (3.1 MPa peak negative pressure) generally caused cavitation and platelet aggregation. Studies by Deng et al. (1996) found they could not cause cavitation in whole blood at up to 5.2 MPa peak negative pressure for 2.5-MHz pulsed ultrasound (0.81% duty cycle), or up to 6.2 MPa peak negative pressure for 4.3-MHz pulsed exposures. HIFU exposures of 1.1-MHz were used in this study and, thus, a lower cavitation threshold is expected. However, cavitation threshold differences may also be attributed to differences between the cell density in whole blood and PRP, because higher cell densities may serve to shield bubbles, as suggested by Brayman and Miller (1993).

Several acoustic mechanisms were considered to explain HIFU-induced platelet activity, including thermal absorption of acoustic energy, cavitation and associated microstreaming, and acoustic streaming and radiation pressure effects. The data for these HIFU-induced platelet activity studies suggest that cavitation, more so than acoustic streaming or thermal effects, plays a role in producing platelet activity in samples of PRP.

The thermal mechanism for cell damage results from exposure to temperatures that tend to denature proteins (i.e., to alter the properties of proteins such that they lose their biologic activity). The temperature required to cause damage is dose-dependent. For example, the time required to kill 90% of Chinese hamster lung fibroblasts is 58 min at 43°C and 27 min at 44°C (ter Haar 1986). The highest temperature reached in any of these trials was 42.7°C, whereas most trials did not rise above 40°C. The maximum duration of exposure was 500 s, so thermal effects are not a likely mechanism to cause platelet activity in these HIFU trials. Because temperature measurements at the focus of the HIFU transducer may lead to artifacts due to absorption by the thermocouple wires in the acoustic field (Waterman and Leeper 1990), the temperature measurements in these studies were taken within the HIFU tank, where the acoustic absorber tends to absorb energy and heat the tank.

Although acoustic streaming and radiation pressure did occur as a result of HIFU exposure, the flow produced in the sample chamber would have to be of sufficient magnitude to shear platelets, thereby activating them. The small size of the platelets (2–4 $\mu$m) requires large shear gradients, which cannot be achieved by

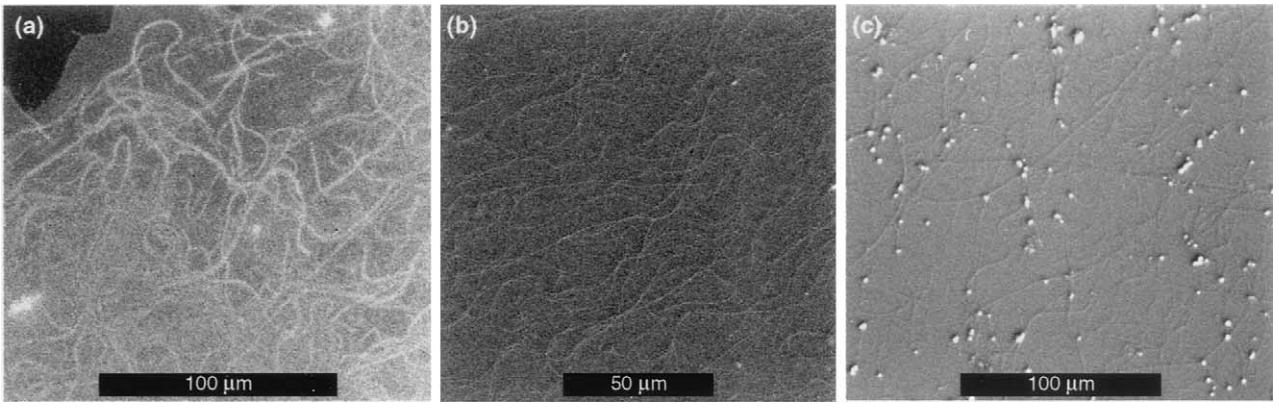


Fig. 9. Adhesion of platelets to a collagen-coated surface, displaying environmental scanning electron microscope images of: (a) control collagen-coated surface in which no HIFU or PRP was applyed, (b) sham collagen-coated surface in which PRP was allowed to contact the surface for 5 min without HIFU exposure, and (c) collagen-coated surface that was exposed to 2 min of 1.1-MHz HIFU at 1000 W/cm$^2$. A few platelets can be seen on the sham collagen-coated surface (b), but many platelets and platelet clumps are attached to the collagen strands in the HIFU exposed sample (c).

acoustic streaming alone in our experimental set-up. Studies by Anderson et al. (1978) used a rotational viscometer to expose PRP to 5 min of shear. They found that a shear stress of 160 dyne/cm$^2$ was required to produce shear-induced platelet activation, with shear stress threshold increasing as exposure time decreased. With estimated platelet velocities of less than 10 cm/s, the geometry of our sample chambers is such that production of shear stresses of this magnitude is unlikely.

Our main hypothesis was that HIFU causes cavitation, which can lead to ripping or shearing of a small number of platelets whose contents are then released, serving to activate other platelets. Ripping of platelets may occur due to inertial cavitation, the violent collapse of a bubble, which can be generated as a result of HIFU exposure. Also, microstreaming can occur near the surface of bubbles undergoing stable cavitation. The cavitation measured in these experiments is most likely inertial cavitation. Stable cavitation is more difficult to identify because it does not efficiently produce large amounts of broadband noise, although spectral analysis of cavitation data can provide information on generation of harmonics that may allow distinction between stable and inertial cavitation. Microstreaming that occurs close to oscillating bubbles may produce shear stresses capable of shearing or ripping apart platelets that are in the vicinity of the bubble. Microstreaming produced by an oscillating wire, simulating a cavitating bubble, has been shown to produce sufficient hydrodynamic shear stress to disrupt platelets (Williams et al. 1974), whereas 2.1-MHz diagnostic ultrasound exposure caused microstreaming, which led to platelet aggregation formation around gas-filled pores *in vitro* (Miller et al. 1979). *In vivo* studies have also shown that acoustic microstreaming produced by a wire oscillating at 20 kHz can create platelet aggregates in murine blood vessels (Williams 1977), and vigorous streaming from 1-MHz ultrasound applied to a micropipette can cause thrombi to form in mouse mesenteric vessels (Frizzel et al. 1986). However, the former studies used simulated cavitation or fixed bubbles. Platelets in the current study would have to be in very close proximity to be activated by a bubble that is oscillating such that it produces microstreaming.

In the 10 s HIFU exposures, HIFU-induced streaming occurred for only 10 s, followed by stirring with a magnetic stir bar. In these trials, cavitation seems to be the dominant mechanism, because aggregation occurred only in those samples that produced cavitation at RCD greater than 180. HIFU-induced streaming without cavitation, followed by stirring, did not induce platelet aggregation. In some of the 10 s HIFU exposures, microaggregates appeared and then reverted back to individual platelets (disaggregation), displaying a response similar to the "subthreshold" activation of platelet aggregation observed by Chater et al. (1977) and Williams et al. (1978).

An important issue to consider when discussing cavitation is that most biologic samples tend to contain very few cavitation nuclei unless they are excessively handled (lung and bowel being exceptions). Samples which are free of dirt and contamination have a higher cavitation threshold than those which are not (Apfel 1970; Greenspan and Tschiegg 1967). Centrifuging, washing and diluting, as well as pipetting, can all add air bubbles and thus potential cavitation nuclei to a sample. Samples in these studies were handled as little as possible in an effort to decrease the amount of impurities present in samples. Randomly trapped impurities in the

sample may contribute to the variation in the cavitation threshold from sample to sample and result in the low correlation between intensity and cavitation.

High-intensity focused ultrasound has been shown to adhere platelets to a collagen-coated surface and to produce platelet activation and aggregation that tends to increase with intensity. Cavitation measurements taken during aggregation trials show a closer relationship between cavitation and aggregation than between intensity and aggregation. We conclude that cavitation seems to play a role in inducing platelet activity, whereas shearing of the platelet by acoustic streaming is less likely to be a mechanism of platelet activity. These biologic effects suggest that HIFU might be useful as a noninvasive method to achieve faster clot formation and reduce blood loss *in vivo*.

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