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Investigations into pulsed-high intensity focused ultrasound enhanced delivery: Preliminary evidence for a novel mechanism

Hilary A. Hancock^{*}, Lauren H. Smith^{*}, Julian Cuesta^{*}, Amir K. Durrani^{*}, Mary Angstadt^{*}, Mark L. Palmeri^Φ, Eitan Kimmel^Ψ, and Victor Frenkel^{*}

^{*} Diagnostic Radiology Department, Clinical Center, National Institutes of Health, Bethesda, MD, USA 20892

^Φ Department of Biomedical Engineering, Duke University, Durham, NC, USA 27708

^Ψ Department of Biomedical Engineering, Technion-Israel Institute of Technology, Haifa, Israel 32000

Abstract

Pulsed-high intensity focused ultrasound (HIFU) exposures without ultrasound contrast agents have been used for non-invasively enhancing the delivery of various agents to improve their therapeutic efficacy in a variety of tissue models in a non-destructive manner. Despite the versatility of these exposures, little is known about the mechanisms by which their effects are produced. In this study pulsed-HIFU exposures were given in the flank muscle of mice, followed by the administration a variety of fluorophores, both soluble and particulate, by local or systemic injection. In vivo imaging (whole animal and microscopic) was used to quantify observations of increased extravasation and interstitial transport of the fluorophores as a result of the exposures. Histological analysis indicated that the exposures caused some structural alterations such as enlarged gaps between muscle fibers. These effects were consistent with increasing the permeability of the tissues; however they were found to be transient and reversed themselves gradually within 72 hrs. Simulations of radiation force induced displacements and the resulting local shear strain they produced were carried out to potentially explain the manner by which these effects occurred. A better understanding of the mechanisms involved with pulsed-HIFU exposures for non-invasively enhancing delivery will facilitate the process for optimizing their use.

Keywords

Pulsed-high intensity focused ultrasound; muscle; extravasation; interstitial transport; radiation force displacements; shear strain

Address correspondence to: Victor Frenkel, Ph.D., Radiology and Imaging Sciences, Clinical Center, National Institutes of Health, Building 10, Room 1N306a, 10 Center Drive, Bethesda, MD 20892, Tel: (301)402-5710, Fax: (301)435-2714, Email: vfrenkel@cc.nih.gov.

(Hynynen, 2007; Kimmel, 2006) (Clement, 2004; Dromi et al., 2007; Frenkel et al., 1999; Kennedy, 2005) (Baker et al., 2006; Baker et al., 2007; Bercoff et al., 2004; Clarkson and Hubal, 2002; Deng et al., 2004; Dittmar et al., 2005; Dreher and Chilkoti, 2007; Fatemi and Greenleaf, 1999; Frenkel, 2008; Frenkel et al., 2006a; Frenkel et al., 2000a; Frenkel et al., 2000b; Frenkel et al., 2006b; Fry, 1968; Fry et al., 1958; Geronilla et al., 2003; Hama et al., 2007; Hynynen et al., 2005; Jain, 1999; Jensen and Svendsen, 1992; Jones et al., 1997; Khaibullina et al., 2008; Kishino and Yanagida, 1988; Kong et al., 2001; McDannold et al., 2008; Miller et al., 2002; Nightingale et al., 2001; O'Brien, 2007; Palmeri et al., 2006; Palmeri et al., 2005; Palmeri et al., 2008; Patel et al., 2008; Peake et al., 2005; Pinton et al., 2006; Poff et al., 2008; Proske and Allen, 2005; Sarvazyan et al., 1998; Stone et al., 2007)

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INTRODUCTION

The vast majority of therapeutic ultrasound applications involve the generation of high temperatures for thermally ablating tissue by the process of coagulative necrosis. The feasibility of this type of treatment for human beings was first demonstrated half a century ago (Fry et al., 1958). Today, high intensity focused ultrasound (HIFU) is being used in the clinic for ablating tumors such as those in the prostate, and uterine fibroids (Kennedy, 2005). The attraction of using ultrasound in this manner is that the energy can be focused deep inside the body with no effects on the intervening tissue, and accurately targeted using various modes of image guidance such as diagnostic ultrasound and magnetic resonance imaging (Clement, 2004).

By providing HIFU exposures using short pulses and low duty cycles, the overall rate of energy deposition for an exposure can be substantially lowered so that temperature elevations will be minimal (i.e. a few degrees Celsius) and, consequently, non-destructive. Instead of generating heat, the ultrasound energy can interact with the tissues through non-thermal mechanisms (Frenkel, 2008). Acoustic cavitation is the most important non-thermal ultrasound mechanism for creating changes in biological tissues, including enabling a number of prominent drug delivery applications presently in development (Kimmel, 2006). These include drug and gene delivery by the process of sonoporation, where bubble collapse at a cell surface can temporarily and non-destructively increase membrane permeability to enable uptake of DNA and other agents (Miller et al., 2002). Conversely, the activity of stably oscillating bubbles (i.e. non-inertial cavitation) is thought to be responsible for enhancing the permeability of blood vessels, through the generation of local streaming and consequent shear that can open up gaps between endothelial cells. This phenomenon is presently being pursued for opening the blood brain barrier (BBB) for the delivery of various macromolecules to the brain that would normally be unable to extravasate (Hynynen, 2007). Both sonoporation and opening of the BBB are typically carried out with the addition of ultrasound contrast agents (UCAs) prior to exposures in order to improve the performance of the effects associated with cavitation activity, as well as lower the intensity threshold for inducing them (Kimmel, 2006). Furthermore, their effects are transient on low time scales, being just a few seconds for sonoporation and a number of hours for BBB opening.

In the past few years we have shown how pulsed-focused ultrasound exposures can be used without UCAs for enhancing the delivery of a variety of therapeutically important agents for the treatment of cancer and other diseases, and in that way improve the efficacy of those treatments. Here, ultrasound exposures are first given and then followed by either local or systemic administration of the agents. This type of treatment was shown to enhance tumor growth inhibition for local administration of plasmid DNA encoding for tumor necrosis factor alpha (TNF α) (Quijano et al., 2005), as well as for an attenuated adenovector expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Patel et al., 2007). In regards to systemic administration, the procedure also enhanced tumor growth inhibition of the proteasome inhibitor bortezomib (Poff et al., 2008). Enhanced systemic delivery of a radio-labeled monoclonal antibody to tumors was also found with the same exposures (Khaibullina et al., 2008), as was plasmid DNA encoding for the reporter gene - green fluorescent protein (GFP) (Dittmar et al., 2005). In studies carried out in whole blood clots, the exposures were found to improve the binding and penetration of tissue plasminogen activator (tPA) (Stone et al., 2005). Therapeutic studies showed that combining pulsed-HIFU with tPA administration could also improve the rate of thrombolysis both in vitro (Frenkel et al., 2006b) and in vivo (Stone et al., 2007) in comparison to administering tPA on its own.

We recently carried out a study in murine muscle, looking at systemic delivery of fluorescently labeled polystyrene nanospheres as a surrogate for drug delivery vehicles. Pulsed-HIFU exposures were found to enhance the delivery of the nanospheres in the targeted tissue, where the enhancing effects were observed to last for at least 24 hrs. Experiments were also carried out to demonstrate that a thermal mechanism was not occurring to produce these effects (O'Neill et al., 2008). In the present study, we continued to develop this model for investigating the effects of pulsed-HIFU on enhancing systemic delivery. Experiments were also carried on the effects on interstitial transport, and we increased the time span for monitoring the reversibility of the enhancement, including a time-course histological study where delivery enhancement was correlated with structural changes created in the tissue. Modeling of displacement fields produced by radiation forces, and stress/strain fields generated by them, was also performed as the basis of a proposed novel non-thermal and non-cavitational ultrasound mechanism potentially involved in this type of delivery enhancement.

MATERIALS AND METHODS

Animals

All animal work was performed in accordance with an approved animal study protocol and in strict compliance to National Institutes of Health Clinical Center Animal care and Use Committee guidelines and regulations. Female C3H mice, aged 23 to 30 weeks, weighing 30 to 35 gr. were used for all studies.

Fluorophores

Four different fluorophores were used in this study. Their respective characteristics and routes of administration are listed in table 1. Lectin (Vector Labs, Burlingame, CA) was administered systemically at its stock concentration of 1 mg in 100 μl^{-1} . For systemic administrations, polystyrene nanospheres (Molecular Probes, Eugene, OR) were also administered in their stock form. To conserve an overall constant volume of nanospheres, the 100 nm and 200 nm nanosphere suspensions had an approximate concentration of 10^{13} ml^{-1} and 10^{12} ml^{-1} , respectively. For local administrations, bovine serum albumin (BSA) and 100 nm nanospheres were co-administered in a pre-prepared cocktail. Individual vials of BSA conjugated to Alexa fluor 485 (Molecular Probes, Eugene, OR) were reconstituted in 1 ml of saline to produce a stock concentration of 5 mg ml^{-1} . 1 ml of the BSA stock solution was mixed with 1 ml of the 100 nm nanospheres. The resulting cocktail possessed a BSA concentration of 2.5 mg ml^{-1} and nanosphere concentration of approximately $5 \times 10^{12} \text{ ml}^{-1}$.

HIFU system

A custom built, image guided, HIFU system modified from a Sonoblate 500 (Focus Surgery, Indianapolis, IN) was used to carry out the pulsed-HIFU exposures. The probe was comprised of both a spherical, concave therapeutic transducer (1 MHz; 5 cm diameter; focal length of 4 cm) and a collinear imaging transducer (10 MHz; 8 mm aperture). The maximum power available to the therapeutic transducer was 120 W. The focal zone was in the shape of an elongated ellipsoid with an axial length (-3 dB) of 7.2 mm and a radial dimension (-3 dB) of 1.38 mm.

HIFU exposures

HIFU exposures were carried out as previously described (Dittmar et al., 2005; Frenkel et al., 2006a; Khaibullina et al., 2008; O'Neill et al., 2008). In short, the mice were anesthetized for the duration of the exposure process using inhalation isoflurane (2%; O_2 1 L hr^{-1}). Twenty four hours prior to exposures, the thigh muscle of the mouse was prepared, by shaving the region and applying NairTM depilation cream for 1 min, followed by a rinse in water.

Immediately prior to HIFU exposures, the region was covered with ultrasonic coupling gel to exclude trapped gas bubbles. (Note, the contra-lateral (control) side of each mouse received the exact same preparatory treatment). The mouse was secured in a holder attached to a three dimensional stage and placed upright in a tank of degassed water maintained at 36 °C, where the mouse's head was kept above the water line in the anesthesia cone. The stage allowed for exact positioning of the mouse limb directly opposite the transducer. Using the graphic user interface of the HIFU system and the imaging transducer, the limb was position symmetrically within the focal zone of the transducer in the X (lateral) and Y (vertical) planes, where these both are perpendicular to the direction of the propagating wave (see fig. 1). For treatment planning, eight individual exposures were evenly spaced in a grid (2 mm in each dimension) for a region of treatment of 4 × 8 mm. Because the axial dimension of the focal zone was greater than the depth of the tissue being treated there was no need to raster in the Z plane (parallel to the direction of the propagating wave). The following exposure parameters were used. Total acoustic power: 40 W; pulse repetition frequency: 1 Hz; duty cycle: 5% (50 ms on/950 ms off). Taking into account the cross-sectional area of the focal zone of the HIFU beam, this produced a spatial average intensity (I_{sa}) of 2660 W cm⁻² (peak pressure amplitude = 8.95 MPa) during each pulse, and a spatial average, temporal average intensity (I_{sata}) of 133 W cm⁻², over an entire cycle. A total of 100 pulses were given at each raster point. With this configuration, an exposure lasted 13 min. and 18 sec. The experimental setup and treatment planning are shown in fig. 1. Note, the power and intensities stated above were based on free-field measurements in degassed water using the radiation force balance measurement technique. Because there were no intervening tissues between the face of the transducer until the focal region (where the targeted muscle was located) these values were assumed to be the approximately those existing in-situ during the treatments.

Systemic delivery of lectin and nanospheres

Immediately after HIFU exposures, mice (n = 5) were given an intravenous tail vein injection of fluorescent lectin (100 µl) at the concentration mentioned above. One hour later the animal was euthanized and the skin over the treated and control regions removed. The animal was positioned onto the stage of an inverted fluorescent microscope (Leica Microsystems, Bannockburn, IL), with the region of interest (control or treated with pulsed-HIFU) placed in contact with a glass cover slip that was adhered to a cell culture dish attached to the stage. Representative images were captured at 100× and 200× with a standard digital camera, and saved in TIFF format. For the visual characterization of delivery of the 100 nm fluorescent nanospheres, mice (n = 10) were similarly given an intravenous tail vein injection (100 µl) immediately after the pulsed-HIFU exposures. Digital images were captured (as above) immediately after treatment (n = 5) and 24 hrs. post-treatment (n = 5).

60 mice were used for a time course study. These were given HIFU exposures, after which half received intravenous injections of 100 nm nanospheres and the other half received the 200 nm nanospheres. For each of the nanosphere diameters, the mice were divided into three equally sized groups (n = 10). One group received injections immediately after the exposures and the remaining two groups at 24 & 48 hrs. post-HIFU. For all groups, digital images were captured (as above) at 24 hrs. post-treatment for both control and HIFU treated tissues. For this experiment, three representative images were taken from each mouse (control & HIFU) for each treatment (a typical image is seen in fig. 3 at 24 hrs.). All images were processed using Image-Pro Plus (MediaCybernetics, Bethesda, MD), where the automatic threshold was set to include bright pixels as objects of interest. The backgrounds of the images were first flattened, which made the black background of the image more uniform. This allowed for a more accurate differentiation between the background and the brighter pixels that represented the nanospheres. Using built-in features of the program, groupings of NPs in the images were then counted, labeled, and measured for area (in pixels). The areas of the pixel groupings were

summed together; where the sum provided a measure of the nanospheres' projected area in the tissue. This value was used as a relative nanosphere concentration in the tissue for treatments using the 100 nm nanospheres (all images possessed an equal number of overall pixels). For treatments using the 200 nm nanospheres, this sum was divided by 4 to adjust for the 2 fold increase in diameter, and hence a 4 fold increase in project surface ($A_{200}/A_{100} = \pi[R_{200}]^2/\pi[R_{100}]^2 = [100]^2/[50]^2 = 4$). For each treatment, a mean value of the three regions was calculated and then these were pooled and presented as the group mean.

Local delivery of albumin and nanospheres

Immediately following HIFU exposure, 20 μ l of the cocktail of fluorescent albumin and 100 nm nanospheres (at the concentration described above) was injected into the treated region of the right limb and the non-treated left hind limb using a 30 G needle. Thirty minutes post HIFU the animals (n = 10) were euthanized and the skin over the treated areas was removed. *In vivo* images of the entire treated areas were then captured for each fluorophore using a Maestro multi-spectral imager (CRI, Cambridge, MA). As previously described (Hama et al., 2007) the Maestro allows for background fluorescence (determined from regions without identifiable positive signal from the administered fluorophores) to be subtracted from regions where positive signal is observed, producing clear and distinct images of only those fluorophores. Images were saved in TIFF format and imported in to Adobe Photoshop® (San Jose, CA) where the total area of the projected signal of each fluorophore was determined. For each treatment the values for each fluorophore were pooled and presented as the group mean.

Histology

In a separate experiment 20 mice were given the same HIFU exposures as in the previous experiments. The mice were divided into 4 groups (n = 5) and were euthanized immediately after treatment, and at 24, 48 & 72 hrs. post-treatment. Both limbs were then removed from the animals and placed in 10% formalin. After 24 hrs. the treated and control tissue was then excised and processed for light microscopy. The tissue was dehydrated in increasing concentrations of ethanol (50 to 100%), and embedded in paraffin. Sections parallel to the long axis of the muscle fibers were cut and mounted on glass slides. These were then deparaffinized in xylene, and stained with hematoxylin and eosin. Slides were observed using a standard brightfield microscope ((Leica Microsystems, Bannockburn, IL) at 200 $\times$ and 400 $\times$, and representative digital images were captured. Control samples were taken randomly from animals at the different time points. Note, a 24 hr time period was used between initial incubation of the entire limbs in formalin and removal of the actual tissue for analysis in order to properly fix the tissue and not create structural artifacts by the removal of the tissue itself. This technique was found to be successful by observations of the control tissues.

Simulations

Field II, a linear acoustic field simulation program (Jensen and Svendsen, 1992) was used to simulate the three dimensional intensity fields generated by the HIFU exposures for an amplitude attenuation of $\alpha = 0.058$ np/cm/MHz. The acoustic radiation force field associated with this intensity distribution was calculated over a three dimensional, quarter symmetry mesh extending $3.85 \times 3.85 \times 7.0$ cm with cubic elements with a node spacing of 0.367 mm. The radiation force was computed as $F = 2 \alpha I/c$ (O'Brien, 2007), where α is the amplitude attenuation coefficient of the medium, I is the average intensity over an element volume at a given node in the mesh, and c is the medium's sound speed (1540 m/s). The mechanical response of an isotropic, linear, elastic solid to the radiation force excitation associated with pulsed-HIFU exposures was then simulated for a single HIFU pulse using finite element methods (FEM). This mechanical acoustic radiation force modeling approach has been previously validated in tissue-mimicking phantoms and has been shown to accurately model

the soft tissue dynamics associated with acoustic radiation force excitations (Palmeri et al., 2005; Palmeri et al., 2006a; Palmeri et al., 2008). For this study, the material was modeled as non-attenuating water for the first 35 mm from the piston face, and a linear, elastic solid with a Young's modulus (E) of 27 kPa and a Poisson's ratio (ν) of 0.499 (nearly incompressible) deep to that to approximate the properties of skeletal muscle, while neglecting the effects of anisotropy given the relatively long durations of insonification for each HIFU pulse. The finite element analysis was performed using LS-DYNA (Livermore Software Technology Corporation, Livermore, CA) using an explicit, time-domain algorithm with single quadrature elements using Flanagan-Belytschko integration stiffness form hourglass control. Post-processing of the displacement and strain fields was performed using LS-PRESPOST2 (Livermore Software Technology Corporation, Livermore, CA) and custom-written scripts in Matlab (MathWorks, Natick, MA) and Perl.

RF Data Acquisition and Displacement Estimation

The displacements induced by the insonifications were experimentally measured for comparisons with the simulation data. Radio frequency (RF) data were acquired with the collinear imaging transducer operating at 10 MHz, with a pulse repetition frequency of 2.54 kHz and a 50 MHz sampling rate. Data were acquired from mice immediately after being euthanized to reduce motion artifacts. Using a separate group of mice ($n = 6$), RF data were acquired at powers of 30, 40 & 50 W, with 4–6 different acquisitions being performed at each power. Displacements were estimated using 1-D normalized cross correlation on the RF data. The RF data were up-sampled to 100 MHz using a 1.1 mm correlation windows using a symmetric $\pm 500 \mu\text{m}$, non-overlapping search window. Raised cosine correlation coefficient interpolation was used to achieve 1 μm displacement resolution (Pinton 2006). Displacement estimates with correlation coefficients < 0.7 were removed from the analysis due to excessive motion artifacts.

Statistical analysis

A paired student's t-test (JMP, SAS Institute, Cary, NC) was used to determine if significant differences occurred in relative nanosphere concentrations between treated and control groups for each nanosphere diameter, and at each time point, in the time course study for the systemic deliveries. The same test was used to determine if significant differences occurred in the area of distribution between treated and control groups for each fluorophore for the local deliveries. A P-value of 0.05 or less was considered as significant.

RESULTS

Systemic administration of fluorescent lectin

Animals were given systemic administration of fluorescently labeled lectin after pulsed-HIFU exposures. Images captured *in vivo* at low magnification showed markedly brighter signals in the HIFU treated muscle compared to controls, where the fluorescence also occurred in the extra-vascular space adjacent to the capillaries. In images captured at high magnification, capillaries in control tissue appeared finely delineated; however in the HIFU treated tissue these were often found to be broader and less delineated in the radial dimension. Representative images appear in fig 2.

Systemic administration of fluorescent nanospheres

Animals were given systemic administration of fluorescently labeled nanospheres (100 nm) following pulsed-HIFU exposures. Images captured *in vivo* immediately after treatment showed the nanospheres to be restricted to the vasculature in both control and treated tissues; however, regions in the treated tissues also had much brighter aggregations of fluorescent

signals that appeared broader than the confines of the capillaries. Nanospheres were no longer visible in the control tissues at 24 hrs post-treatment. In the treated tissue, the nanospheres now appeared to be less clumped, and more uniformly distributed throughout the tissue. Representative images appear in fig 3.

Animals were given systemic administrations of fluorescently labeled nanospheres (100 nm or 200 nm) at 0, 24, and 48 hrs. post-HIFU exposures. *In vivo* images were captured 24 hrs. post-administration and visible nanosphere density was quantified and represented as the relative concentration. At each one of the time points, a greater concentration was observed for the 100 nm nanospheres compared to the 200 nm nanospheres. In general, a trend was observed for both nanosphere sizes, where a decrease in concentration was found with increasing delay between HIFU exposures and administrations. Significant differences between treated and untreated tissue were found at each time point for both nanospheres sizes ($p < 0.05$, $n = 10$). Results appear in fig 4.

Local administration of fluorescent albumin and nanospheres

Fluorescently labeled albumin and nanospheres were co-administered locally by direct injection in treated (HIFU) and untreated (control) muscle immediately after HIFU exposures. *In vivo*, multi-spectral images of the flanks were captured for the albumin and nanospheres 30 min. after administrations. In control tissues, both fluorophores were found to accumulate at the end of the needle track, which was not seen in treated tissues. Nanosphere distribution was also observed to be noticeably more restricted than the albumin in controls, possessing on average, one fourth the spatial distribution. Although a trend of increased distribution of the albumin was observed for HIFU treated tissue compared to controls, a significant difference was not found. A significant difference was found for the nanosphere distribution, which was on average 65% higher in the HIFU treated tissue ($p = 0.01$, $n = 10$). When the nanosphere distribution was normalized to that of the albumin, it was still found to be significantly higher in the HIFU treated tissue ($p = 0.04$, $n = 10$). Results appear in figure 5.

Histology

Histological sections were prepared from control and HIFU treated tissues. Those treated with HIFU were collected immediately after treatment, as well as at 24, 48 and 72 hrs post-treatment. Enlarged gaps between muscle fibers were observed in HIFU treated tissues compared to controls. These were observed to be largest immediately after treatment, where a trend of decreasing gap size was observed over time. Tissues at 72 hrs appeared similar to controls. Disruption of the connective tissue was found between the enlarged gaps, especially immediately after treatment. Some capillaries in the HIFU treated tissue were also found to be disrupted, and free red blood cells were observed between the fibers. These were found primarily immediately after treatment and to a lesser degree at 24 hrs., but not at the later time points. Larger blood vessels were not found to be affected at all by the exposures. Representative images appear in figure 6.

Simulations

The displacements and strain fields generated during a HIFU pulse were able to achieve a steady-state deformation due to the relatively long excitation duration (50 ms), as demonstrated in Figure 7 (upper left). In this simulated 27 kPa medium, the steady-state focal point displacement was achieved within 5 ms of the initiation of the pulse and reached a peak magnitude of $\sim 5.5 \mu\text{m}$. After cessation of the pulse, the tissue was able to recover within another 5 ms. The oscillations in the focal point displacement are due to the lack of loss in the simulated purely elastic material and the finite boundaries of the simulated volume of tissue that reflect mechanical waves back into the region of interest later in simulated time. During the pulse, the displacement field is concentrated around the focal zone (Figure 7 – top right: a & b), but after

the pulse, shear waves propagate into the tissue adjacent to the focal zone (Figure 7 – top right: c & d). Note that initially the off-axis extent of the displacement is restricted to < 5 mm during the HIFU pulse, but grows to > 15 mm in the steady-state condition (Figure 7 – lower left). After cessation of the pulse (> 50 ms), the shear wave profiles can be appreciated propagating away from the region of excitation with decaying displacement amplitudes due to geometric spreading through time. Unlike the displacement profiles, the von Mises strains—predominately shear strains—are maximized at the greatest displacement gradients (Figure 7 – bottom right), which remain relatively stable during the HIFU pulse with peaks offset from the axis of symmetry that then travel with the propagating shear waves after cessation of the pulse.

Experimental Displacement Data

Figure 8 shows the experimental displacement data acquired from skeletal muscle *in situ* using a separate group of mice. These data were collected for comparison with the simulated displacements shown in Figure 7. The relaxation from the steady state displacement induced by the 50 ms HIFU exposures (Figure 8 – bottom) is shown for three different HIFU powers (30, 40 & 50 W). Measurements were also taken at 0 W (sham pulse) to demonstrate the noise associated with the procedure. The peak steady-state displacements generated by these different powers are linear as a function of the applied HUFU power (Figure 8 – inset). Error bars represent the variability between different mice over different trials. The relaxation time back to the unperturbed state (0 μ m) is 6–8 ms, compared to ~5 ms in the simulation data.

DISCUSSION

In the present study, pulsed-HIFU exposures were carried out in the flank muscle of mice, followed by either systemic or local administrations of a variety of fluorophores. Investigations on extravasation and interstitial transport were performed using quantitative post analysis of images captured *in vivo*. The muscle was selected as a targeted tissue for a number of reasons. For one, the microvasculature is non-fenestrated, making it sensitive to any effects on extravasation. In a recent study we showed, as found here, that indeed significantly higher numbers of nanospheres could be delivered to the tissue when systemic administrations of the nanospheres followed pulsed-HIFU exposures to the targeted tissue (O'Neill et al., 2008). In a similar manner to these effects on extravasation, the present study also showed that significant increases in interstitial transport could occur as a result of the exposures when the nanospheres were administered locally. Secondly, the structural organization of the parenchyma and microvasculature in muscle is much more regular than that of, for example, tumors, where the microvasculature can be very chaotic and leaky, and factors in the extracellular matrix (e.g. fibrillar collagen) can also inhibit transport (Dreher and Chilkoti, 2007). The more regular structure of the muscle tissue (vasculature and parenchyma), therefore, allowed for a more straight forward and reproducible characterization of the pulsed-HIFU effects on delivery.

In regards to the ultrasound mechanisms for explaining the results obtained in the present study, we have recently shown that in this experimental model (e.g. using the same nanospheres in murine muscle), that a thermal mechanism can unequivocally be ruled out. In that study we provided the equivalent thermal dose produced by the exposures using a non-ultrasound source, and found no increase in the systemic delivery of the nanospheres (O'Neill et al., 2008). We have shown that these pulsed-HIFU exposures typically produce temperature elevations of 4 to 5 °C for an individual treatment of 2 min or less in tumors (Frenkel et al., 2006a; Patel et al., 2008) and in muscle (O'Neill et al., 2008). Temperature elevations such as these are capable of enhancing extravasation of nanoparticles, however, treatments are required to last an hour in order to be effective (Kong et al., 2001). Furthermore, the enhancement will generally last for about 6 hrs until the effects are reversed. O'Neill et al. (2008) showed that the effects of

pulsed-HIFU exposures for enhancing extravasation endure for at least 24 hrs. And here, we present data showing the effects last even longer than 48 hrs. All this evidence combined leads us to believe that a thermal mechanism cannot reasonably be directly involved for producing these effects.

Despite these assertions, it should be mentioned that although increases in temperature from the pulsed-HIFU exposures appear not to be directly contributing to enhancing delivery (as found for pure hyperthermia treatments (see Kong and Dewhirst (1999))), there still exists the potential that the temperature elevations that do occur with these exposures may be playing a more secondary role synergistically with non-thermal mechanisms of ultrasound. Increases in temperature can result in changes in the physiochemical state of cell membranes, causing them to transition from a gel phase to a more fluid phase (i.e. melting). This involves a reduction in the conformational order of the lipid chains, rendering them more susceptible to deformation (Mishima et al., 2001). During this transition dynamic changes also occur in the viscosity, heat capacity, and elastic constant of the lipid membranes (Seifert et al., 1991), which can ultimately have effects on structural characteristics, such as membrane curvature (Schneider et al., 1999). Increases in temperature towards the transitional state of chain melting has, for example, been found to decrease the bending modulus of multilamellar bilayers (Mishima et al., 2001), as well as decrease the shear stiffness of bovine muscle *ex vivo*, as measured with magnetic resonance elastography (Kruse et al., 2000). In light of this evidence, there appears to be the potential for a synergistic role of temperature elevations created by the pulsed-HIFU exposures, where these could conceivably enhance the degree of mechanically induced effects.

After a thermal mechanism, acoustic cavitation is the next best understood mechanism of ultrasound, as well as being the most broadly described for enhancing delivery (Frenkel, 2008). For drug and gene delivery applications that rely on acoustic cavitation, ultrasound contrast agents are typically added for enhancing cavitation activity and its consequent effects, as well as for reducing the intensity threshold at which cavitation will occur (Kimmel, 2006). This includes inertial cavitation for delivering agents into intact cells via sonoporation (Miller et al., 2002; Deng et al., 2004), and non-inertial cavitation for opening the blood brain barrier (BBB) (McDannold et al., 2008). In regards to the pulsed-HIFU exposures used in the present study, the fact that UCAs were not used did not preclude cavitation as a viable mechanism contributing to enhancing delivery, at least in the case of extravasation. In both muscle (O'Neill et al., 2008) and in tumors (O'Neill et al., 2006) standard *in vivo* monitoring techniques did detect the presence of both inertial and non-inertial cavitation, however, in studies in tumors, this was found to be restricted to the vascular rich regions, such as the dermis and the tumor periphery (O'Neill et al., 2006). In studies in the muscle, we showed indeed that relatively greater amounts of nanospheres were found closer to the outer surface of the tissue when systemic administrations were used (O'Neill et al., 2008). The contention, however, that cavitation was not the only mechanism responsible for enhancing extravasation becomes more viable when looking at the endurance of the induced effects. In past studies in the muscle we found that enhancement occurred for at least 24 hrs post-treatment (O'Neill et al., 2008), and in the present study for at least 48 hrs post-treatment. In studies for opening the BBB the enhancing effects are reversed after only a few hours, even when UCAs are employed (Hynynen et al. 2005).

Further support for a non-cavitation mechanism to be present is provided by the observed effects on interstitial transport. Cavitation activity is much less likely to occur in the interstitium because of a dearth of cavitation nuclei as well as a lack of physical space for bubble oscillation and expansion (Simonin, 1995). In one particular study carried out in tumors, direct injections into the core of tumors of plasmid DNA encoding for tumor necrosis factor alpha (TNF α) was evaluated with and without preceding pulsed-HIFU exposures. Larger regions of necrosis were observed in those that received pretreatment with pulsed-HIFU, which then correlated with

slower growth of tumors, compared to tumors receiving injections only (Quijano et al., 2005). As described above, whereas cavitation activity was detected for the exposures at the periphery of these tumors, it was not found in their cores where the injections were given, and where the beneficial therapeutic effects were consequently observed (O'Neill et al., 2006).

The unlikelihood of cavitation having a role for enhancing interstitial transport is further supported by the results obtained of the histological analysis. Whereas enlarged gaps between muscle fiber bundles were observed, no damage (e.g. pitting or erosion) was found at the surface of the bundles, that is typical of bubble collapse, and the shock waves and wall direct re-entrant jets that consequently ensue (Frenkel et al., 1999). The enlarged gaps between the fiber bundles, and the disruption of the connective tissue found between them, instead would seem to indicate the actions of a different form of mechanical energy deposition. In the past, we have proposed displacements created by radiation forces generated in the tissue as a possible alternative mechanism to heat and cavitation for creating structural effects in the tissue that could enhance its permeability. This idea was at the time based more on speculation because of the relatively large displacements that would occur (i.e. on the order of 10's of microns), as well as preliminary empirical data indicating a correlation between the degree of displacement generated and the resulting delivery enhancement (Frenkel et al., 2005; Frenkel et al., 2006b).

Acoustic radiation force is a body force that is generated by a propagating acoustic wave as some of its momentum is transferred to its propagation medium through loss mechanisms (primarily absorption in soft tissue) (O'Brien, 2007). These forces can induce small displacements and strains that cannot be visually appreciated, however, the resulting deformations can be measured when the raw radiofrequency (RF) data is processed from an ultrasound scanner (Pinton et al., 2006). This approach of utilizing acoustic radiation force to deform tissue and reconstruct its underlying mechanical properties is specifically utilized by several imaging modalities, including Shear Wave Elasticity Imaging (SWEI) (Sarvazyan et al., 1998), Supersonic Imaging (SSI) (Bercoff et al., 2005), Vibroacoustography (Fatemi et al., 1999), and Acoustic Radiation Force Impulse (ARFI) imaging (Nightingale et al., 2001). While HIFU pulses use lower intensities than some of these imaging modalities, their relatively long duration allows for appreciable mechanical deformations to occur in the tissues, both within and adjacent to the region of excitation (ROE). The tissue dynamics in response to the acoustic radiation force excitation are shear modulated, and the majority of the von Mises strain profiles are dominated by shear strains (Palmeri et al., 2006a). The peak strain occurs along the greatest displacement gradients, which are offset from the axis of symmetry during the HIFU pulse and then propagate with the shear waves during tissue relaxation. The magnitudes of these strains can be modulated by the HIFU pulse focal configurations, with higher frequency pulses at lower f-numbers (focal depth/aperture width) leading to tighter spatial focusing and greater strain magnitudes.

In the present study radiation force measurements were carried out for a number of different exposures, where we found a positive linear relationship between applied power and peak displacement, as predicted for the generation of radiation forces (Palmeri et al., 2005). At the power (40 W) used in the study for the nanosphere delivery experiments, relaxation rates of the tissue back to its pre-pulsed position were comparable for the simulations and measurements. The measured peak displacement, however, was lower than that predicted by the simulations but still on the same order of magnitude. A number of factors could have contributed to this discrepancy. The impedance mismatch between the degassed water (coupling medium) and the skin and muscle would initially contribute to lower acoustic energy in the focal region, where losses can be as high as 50% (Palmeri, unpublished). Additional sources potentially contributing to the disparity between simulated and measured displacements would be the values used in the simulations for the attenuation coefficient of the muscle, as well as its

stiffness and compressibility (Palmeri et al., 2006b). The question we would like to address in this study is whether the radiation-force-related stress and strain in the ROE of our sonications were capable of inducing the observed alterations in tissue structure (i.e. widening the gaps between muscle fiber bundles).

We believe that the alterations were most likely due to disruption of the relatively weak connections between muscle fiber bundles and connective tissue between them, as was observed. In a previous study, we similarly found that shear strain generated from rapidly attenuating ultrasound transverse waves could generate changes at the relatively weak structural elements in the tissue. The effects manifested themselves as enlarged gaps between epithelial cells, where this occurred without damaging the cells themselves. The desmosomes between the cells, however, were also found to be disrupted but not the more robust tight junctions (Frenkel et al., 2000a). The result of producing such effects was similar to the present study (i.e. increased transport), where increased nanoparticle transport through the tissue occurred in the form of both greater distribution and higher rates of effective diffusion (Frenkel et al., 2000b).

Attempts were made to compare effects generated in the muscle in the present study with others in the literature. We found studies that had similar observations of structural changes in skeletal muscle, where these studies dealt with recreating muscle activity of human locomotion and athletic performance, primarily in the form of stretch-shortening contractions (SSC). Histological analysis in these studies similarly showed enlarged gaps between muscle fiber bundles (Jones et al., 1997; Geronilla et al., 2003; Baker et al., 2006; Baker et al., 2007), and in some cases detachment of the extracellular matrix from muscle fibers into the interstitial space (Clarkson and Hubal, 2002). These morphological changes are thought to occur from initial injury, typically to the sarcomeres (Proske and Allen, 2005), which induce inflammation and edema, evident by the enlarged gaps (Baker et al., 2006; Geronilla et al., 2003; Baker et al., 2007). In cases when the degree of the contractions (and hence, the induced damage) is more extensive, by increasing the length at which the contractions are performed (Proske and Allen, 2005) or increasing their overall number of contractions (Baker et al., 2007), neutrophils and macrophages may also be found to have infiltrated into the tissue (Peake et al., 2005). Neither of these cell types was found in histological samples of the present study.

One important dissimilarity between the present study and those on SSC is that the morphological changes described for the latter occur gradually and reach a peak at 48 hrs. post-treatment (Geronilla et al., 2003; Baker et al., 2006; Baker et al., 2007); a time scale typical of an inflammatory process. In the present study the most extensive effects (i.e. gap formation) occurred immediately post-treatment, and were already mostly diminished by 48 hrs. This would seem to further support the proposed mechanism for inducing these effects with pulsed-HIFU exposures (i.e. by mechanically opening up the gaps), as described above, as well as the contention that the exposures themselves did not cause irreversible damage to the tissues. Furthermore, in the SSC studies the fluid was found to preferentially accumulate between the fiber bundles, even though the damage in the tissues that initiated this process occurred at the level of the sarcomeres of the individual fibers. It is, therefore, not unreasonable to infer that indeed the interfaces between the bundles are the relatively weaker link in the tissue, being first to be susceptible to the strain being generated by the pulsed-HIFU exposures.

In regards to direct damage being produced to the muscle fiber bundles themselves from the large displacements generated in the tissues, it is not at all surprising that this was not observed. Previous pulsed-HIFU studies, using similar intensities and rates of energy deposition, also showed no damage in various tumor models, both human (Khaibullina et al., 2008) and murine (Quijano et al., 2005; Dittmar et al., 2005; Frenkel et al., 2006a; Poff et al., 2008). Furthermore, therapeutic studies showed no effects of the exposures alone on growth rates when compared

to untreated controls (Quijano et al., 2005; Frenkel et al., 2006a; Dromi et al., 2007; Poff et al., 2008). In thrombolytic studies using tissue plasminogen activator (tPA), the same pulsed-HIFU exposures alone did not enhance thrombolysis, either *in vitro* (Frenkel et al., 2006b) or *in vivo* (Stone et al., 2007), nor was damage observed to the endothelium of the blood vessels *in vivo* where the clots were treated (Stone et al., 2007).

CONCLUSIONS

In conclusion, the investigations carried out in the present study showed that pulsed-HIFU exposures are capable of generating non-destructive, and reversible, structural changes in the muscle for enhancing both extravasation and interstitial transport of large (i.e. 100 nm) nanospheres, being on the same size scale of liposomal drug carriers and viral vectors. As previously reported by us, these effects appear not to occur directly from a thermal mechanism of ultrasound. Based on some of the evidence provided here (i.e. the relative long reversal process and lack of direct damage to tissue), acoustic cavitation alone does not seem to be the only nonthermal mechanism taking place. Preliminary evidence is also given, making the case for displacements generated by acoustic radiation forces as possible new ultrasound mechanism for delivery enhancement. A better understanding of the mechanisms involved when using pulsed-HIFU for enhancing delivery will facilitate efforts to optimize these types of exposures for this purpose.

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References

- Baker BA, Mercer RR, Geronilla KB, Kashon ML, Miller GR, Cutlip RG. Stereological analysis of muscle morphology following exposure to repetitive stretch-shortening cycles in a rat model. *Appl Physiol Nutr Metab* 2006;31:167–79. [PubMed: 16604135]
- Baker BA, Mercer RR, Geronilla KB, Kashon ML, Miller GR, Cutlip RG. Impact of repetition number on muscle performance and histological response. *Med Sci Sports Exerc* 2007;39:1275–81. [PubMed: 17762360]
- Bercoff J, Tanter M, Fink M. Supersonic shear imaging: a new technique for soft tissue elasticity mapping. *IEEE Trans Ultrason Ferroelectr Freq Control* 2004;51:396–409. [PubMed: 15139541]
- Clarkson PM, Hubal MJ. Exercise-induced muscle damage in humans. *Am J Phys Med Rehabil* 2002;81:S52–69. [PubMed: 12409811]
- Clement GT. Perspectives in clinical uses of high-intensity focused ultrasound. *Ultrasonics* 2004;42:1087–93. [PubMed: 15234170]
- Deng CX, Sieling F, Pan H, Cui J. Ultrasound-induced cell membrane porosity. *Ultrasound Med Biol* 2004;30:519–26. [PubMed: 15121254]
- Dittmar KM, Xie J, Hunter F, Trimble C, Bur M, Frenkel V, Li KC. Pulsed high-intensity focused ultrasound enhances systemic administration of naked DNA in squamous cell carcinoma model: initial experience. *Radiology* 2005;235:541–6. [PubMed: 15798154]
- Dreher MR, Chilkoti A. Toward a systems engineering approach to cancer drug delivery. *J Natl Cancer Inst* 2007;99:983–5. [PubMed: 17596569]
- Dromi S, Frenkel V, Luk A, Traugher B, Angstadt M, Bur M, Poff J, Xie J, Libutti SK, Li KC, Wood BJ. Pulsed-high intensity focused ultrasound and low temperature-sensitive liposomes for enhanced targeted drug delivery and antitumor effect. *Clin Cancer Res* 2007;13:2722–7. [PubMed: 17473205]
- Fatemi M, Greenleaf JF. Vibro-acoustography: an imaging modality based on ultrasound-stimulated acoustic emission. *Proc Natl Acad Sci U S A* 1999;96:6603–8. [PubMed: 10359758]

- Frenkel V, Kimmel E, Iger Y. Ultrasound-induced cavitation damage to external epithelia of fish skin. *Ultrasound Med Biol* 1999;25:1295–303. [PubMed: 10576272]
- Frenkel V, Kimmel E, Iger Y. Ultrasound-induced intercellular space widening in fish epidermis. *Ultrasound Med Biol* 2000a;26:473–80. [PubMed: 10773379]
- Frenkel V, Kimmel E, Iger Y. Ultrasound-facilitated transport of silver chloride (AgCl) particles in fish skin. *J Control Release* 2000b;68:251–61. [PubMed: 10925133]
- Frenkel V, Deng C, Quijano J, Stone MJ, Dromi S, Hunter F, Xie J, Wood BJ, Li KCP, O'Neill BE, Quinn TP. Pulsed-high intensity focused ultrasound (HIFU) exposures for enhanced delivery of therapeutics: mechanisms and applications. *ISTU Symp.* 2005
- Frenkel V, Etherington A, Greene M, Quijano J, Xie J, Hunter F, Dromi S, Li KC. Delivery of liposomal doxorubicin (Doxil) in a breast cancer tumor model: investigation of potential enhancement by pulsed-high intensity focused ultrasound exposure. *Acad Radiol* 2006a;13:469–79. [PubMed: 16554227]
- Frenkel V, Oberoi J, Stone MJ, Park M, Deng C, Wood BJ, Neeman Z, Horne M 3rd, Li KC. Pulsed high-intensity focused ultrasound enhances thrombolysis in an in vitro model. *Radiology* 2006b;239:86–93. [PubMed: 16493016]
- Frenkel V. Ultrasound mediated delivery of drugs and genes to solid tumors. *Adv Drug Deliv Rev* 2008;60:1193–208. [PubMed: 18474406]
- Fry FJ, Ades HW, Fry WJ. Production of reversible changes in the central nervous system by ultrasound. *Science* 1958;127:83–4. [PubMed: 13495483]
- Geronilla KB, Miller GR, Mowrey KF, Wu JZ, Kashon ML, Brumbaugh K, et al. Dynamic force responses of skeletal muscle during stretch-shortening cycles. *Eur J Appl Physiol* 2003;90:144–53. [PubMed: 14504946]
- Hama Y, Urano Y, Koyama Y, Choyke PL, Kobayashi H. Activatable fluorescent molecular imaging of peritoneal metastases following pretargeting with a biotinylated monoclonal antibody. *Cancer Res* 2007;67:3809–17. [PubMed: 17440095]
- Hynynen K. Focused ultrasound for blood-brain disruption and delivery of therapeutic molecules into the brain. *Expert Opin Drug Deliv* 2007;4:27–35. [PubMed: 17184160]
- Hynynen K, McDannold N, Sheikov NA, Jolesz FA, Vykhodtseva N. Local and reversible blood-brain barrier disruption by noninvasive focused ultrasound at frequencies suitable for trans-skull sonications. *Neuroimage* 2005;24:12–20. [PubMed: 15588592]
- Jensen JA, Svendsen NB. Calculation of pressure fields from arbitrarily shaped, apodized, and excited ultrasound transducers. *IEEE Trans Ultrason Ferroelectr Freq Control* 1992;39:262–7. [PubMed: 18263145]
- Jones C, Allen T, Talbot J, Morgan DL, Proske U. Changes in the mechanical properties of human and amphibian muscle after eccentric exercise. *Eur J Appl Physiol Occup Physiol* 1997;76:21–31. [PubMed: 9243166]
- Kennedy JE. High-intensity focused ultrasound in the treatment of solid tumours. *Nat Rev Cancer* 2005;5:321–7. [PubMed: 15776004]
- Khaibullina A, Jang BS, Sun H, Le N, Yu S, Frenkel V, Carasquillo JA, Pastan I, Li KC, Paik CH. Pulsed high-intensity focused ultrasound enhances uptake of radiolabeled monoclonal antibody to human epidermoid tumor in nude mice. *J Nucl Med* 2008;49:295–302. [PubMed: 18199622]
- Kimmel E. Cavitation bioeffects. *Crit Rev Biomed Eng* 2006;34:105–61. [PubMed: 16749890]
- Kong G, Braun RD, Dewhirst MW. Characterization of the effect of hyperthermia on nanoparticle extravasation from tumor vasculature. *Cancer Res* 2001;61:3027–32. [PubMed: 11306483]
- Kruse SA, Smith JA, Lawrence AJ, Dresner MA, Manduca A, Greenleaf JF, Ehman RL. Tissue characterization using magnetic resonance elastography: preliminary results. *Phys Med Biol* 2000;45(6):1579–1590. [PubMed: 10870712]
- McDannold N, Vykhodtseva N, Hynynen K. Effects of acoustic parameters and ultrasound contrast agent dose on focused-ultrasound induced blood-brain barrier disruption. *Ultrasound Med Biol* 2008;34:930–7. [PubMed: 18294757]
- Miller DL, Pislaru SV, Greenleaf JE. Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somat Cell Mol Genet* 2002;27:115–34. [PubMed: 12774945]

- Mishima K, Nakamae S, Ohshima H, Kondo T. Curvature elasticity of multilamellar lipid bilayers close to the chain-melting transition. *Chem Phys Lipids* 2001;110(1):27–33. [PubMed: 11245832]
- Nightingale KR, Palmeri ML, Nightingale RW, Trahey GE. On the feasibility of remote palpation using acoustic radiation force. *J Acoust Soc Am* 2001;110:625–34. [PubMed: 11508987]
- O'Brien WD Jr. Ultrasound-biophysics mechanisms. *Prog Biophys Mol Biol* 2007;93:212–55. [PubMed: 16934858]
- O'Neill BE, Frenkel V, Li KCP, Quinn TP. Pulsed HIFU for enhanced drug delivery: a study of possible mechanisms. *ISTU Symp.* 2006
- O'Neill BE, Vo H, Angstadt M, Li KCP, Quinn T, Frenkel V. Pulsed high intensity focused ultrasound mediated nanoparticle delivery: Mechanisms and efficacy in murine muscle. *Ultrasound Med Biol.* 2008 Dec 9;[Epub ahead of print]
- Palmeri ML, Sharma AC, Bouchard RR, Nightingale RW, Nightingale KR. A finite-element method model of soft tissue response to impulsive acoustic radiation force. *IEEE Trans Ultrason Ferroelectr Freq Control* 2005;52:1699–712. [PubMed: 16382621]
- Palmeri ML, McAleavey SA, Fong KL, Trahey GE, Nightingale KR. Dynamic mechanical response of elastic spherical inclusions to impulsive acoustic radiation force excitation. *IEEE Trans Ultrason Ferroelectr Freq Control* 2006a;53:2065–79. [PubMed: 17091842]
- Palmeri ML, McAleavey SA, Trahey GE, Nightingale KR. Ultrasonic tracking of acoustic radiation force-induced displacements in homogeneous media. *IEEE Trans Ultrason Ferroelectr Freq Control* 2006b; 53:1300–1313. [PubMed: 16889337]
- Palmeri ML, Wang MH, Dahl JJ, Frinkley KD, Nightingale KR. Quantifying hepatic shear modulus in vivo using acoustic radiation force. *Ultrasound Med Biol* 2008;34:546–58. [PubMed: 18222031]
- Patel PR, Wood BJ, Frenkel V, Nguyen D. Enhancement of AdVgTRAIL Gene Therapy using Pulsed-High Intensity Focused Ultrasound (HIFU) in a Human Esophageal Carcinoma Model in Mice. *RSNA Symp.* 2007
- Patel PR, Luk A, Durrani AK, Dromi S, Cuesta J, Angstadt M, et al. In vitro and in vivo evaluations of increased effective beam width for heat deposition using a split focus high intensity ultrasound (HIFU) transducer. *Int J Hyperthermia* 2008;1–13. [PubMed: 18214764]
- Peake J, Nosaka K, Suzuki K. Characterization of inflammatory responses to eccentric exercise in humans. *Exerc Immunol Rev* 2005;11:64–85. [PubMed: 16385845]
- Pinton GF, Dahl JJ, Trahey GE. Rapid tracking of small displacements with ultrasound. *IEEE Trans Ultrason Ferroelectr Freq Control* 2006;53:1103–17. [PubMed: 16846143]
- Poff JA, Allen CT, Traughber B, Colunga A, Xie J, Chen Z, Wood BJ, van Waes C, Li KC, Frenkel V. Pulsed High-Intensity Focused Ultrasound Enhances Apoptosis and Growth Inhibition of Squamous Cell Carcinoma Xenografts with Proteasome Inhibitor Bortezomib. *Radiology* 2008;8:485–91. [PubMed: 18574138]
- Proske U, Allen TJ. Damage to skeletal muscle from eccentric exercise. *Exerc Sport Sci Rev* 2005;33:98–104. [PubMed: 15821431]
- Quijano J, Colunga A, Xie J, Frenkel V, Li KCP. Enhanced Regression in a Squamous Cell Carcinoma Murine Tumor Model Using Pulsed-High Intensity Focused Ultrasound (HIFU) and Naked TNF- α Plasmid. *SMI Symp.* 2005
- Sarvazyan AP, Rudenko OV, Swanson SD, Fowlkes JB, Emelianov SY. Shear wave elasticity imaging: a new ultrasonic technology of medical diagnostics. *Ultrasound Med Biol* 1998;24:1419–35. [PubMed: 10385964]
- Schneider MF, Marsh D, Jahn W, Kloesgen B, Heimburg T. Network formation of lipid membranes: triggering structural transitions by chain melting. *Proc Natl Acad Sci U S A* 1999;96(25):14312–14317. [PubMed: 10588702]
- Seifert U, Berndt K, Lipowsky R. Shape transformations of vesicles: Phase diagram for spontaneous-curvature and bilayer-coupling models. *Phys Rev A* 1991;44(2):1182–1202. [PubMed: 9906067]
- Simonin JP. On the mechanisms of in vitro and in vivo phonophoresis. *J Control Release* 1995;33:125–141.
- Stone MJ, Frenkel V, Oberoi J, Horne M 3rd, Li KC, Wood BJ. Pulsed-High Intensity Focused Ultrasound (HIFU)-enhanced Thrombolysis in Vitro: Proof of Concept and Investigation of Mechanism. *RSNA Symp.* 2005

Stone MJ, Frenkel V, Dromi S, Thomas P, Lewis RP, Li KC, Horne M 3rd, Wood BJ. Pulsed-high intensity focused ultrasound enhanced tPA mediated thrombolysis in a novel in vivo clot model, a pilot study. *Thromb Res* 2007;121:193–202. [PubMed: 17481699]

Fig. 1.

Experimental setup for HIFU exposures. (Upper left) Water tank with probe and animal holder inserted vertically and attached to 3D stage. (Upper right) Zoom in of hatched region in upper left image showing the transducer positioned directly across from the mouse limb being targeted. Note, the head of the mouse is above the water line. (Bottom) B-mode ultrasound scans from the graphic user interface showing how the targeted muscle is positioned within the focal zone of the transducer. Raster points for treatment planning are added after animal positioning and image acquisition.

Fig. 2.

Representative *in vivo* images captured of fluorescently labeled lectin administered systemically in treated (HIFU) and untreated (control) muscle. Imaging was carried out immediately after treatment. Lower magnification images (top) show overall greater levels of signal in the treated tissue. In higher magnification images (bottom) capillaries appear to be broader and less delineated in the treated tissue compared to controls. Arrows indicate capillaries. Bar = 50 μ m.

Fig. 3.

In *vivo* images captured at early and late time points of fluorescently labeled nanospheres (100 nm) administered systemically in treated (HIFU) and untreated (control) muscle. At 0.5 hrs nanospheres can be seen restricted to the vasculature in the untreated tissue (arrows); however substantial aggregations occur in the treated tissue that appeared broader than the confines of the capillaries. At 24 hrs nanospheres are no longer visible in the untreated tissue, and are now more evenly distributed in the treated tissue. Bar = 50 μ m.

Fig. 4.

Relative nanoparticle concentration in treated and untreated muscle, where a systemic administration of either 100 nm or 200 nm nanospheres was given at 0, 24, and 48 hrs. post-HIFU exposure. Three representative vivo images were captured for each tissue analyzed, and standard image processing techniques were used for quantitating fluorescent signal density. Greater relative concentration was observed for the 100 nm nanospheres compared to the 200 nm nanospheres at each of the time points. A general trend was observed for both nanospheres sizes of decreasing concentration with increasing delay between HIFU exposures and administrations. Significant differences between treated and untreated tissue were found at each time point for both nanospheres sizes ($p < 0.05$, $n = 10$).

Fig. 5.

Distributions of fluorescently labeled albumin (A) and nanospheres (N) co-administered locally by direct injection in treated (HIFU) and untreated (control) muscle. (Upper images) *In vivo* images of the flanks captured for the albumin and nanospheres using multi-spectral imaging. Vertical arrows indicate the site of injection. Rectangles indicate region of treatment of the HIFU exposures. Horizontal arrows show accumulation of the fluorophores in the untreated tissue, at the tip of the injection, being noticeably greater than in the treated tissue. Bar = 2 mm. (Lower left) Spatial distribution of both fluorophores in the treated and untreated tissue. A significant increase in distribution of the nanospheres was found in the treated tissue compared to untreated ($p = 0.01$, $n = 10$). Although a trend of greater distribution of the albumin was also observed in the treated tissue, this was not significantly different than in the controls. (Lower right) Normalization of nanosphere distribution to that of the albumin. Normalized distribution in the HIFU treated tissue was significantly greater than in the untreated muscle ($p = 0.04$, $n = 10$).

Fig. 6.

Representative histological sections of treated (HIFU) and untreated (control) muscle stained with hematoxylin and eosin. (Upper images) Tissues were sampled at 0, 24, 48 and 72 hrs post-HIFU ($n = 5$). Arrows indicate enlarged gaps between the muscle fibers in the treated tissue (compared to controls), which appear largest at 0 hrs and tend to decrease in size over time. (Lower images) Sections of samples taken in treated tissue at 0 hrs. (a) Large blood vessels (arrows) were not found to be disrupted by the exposures; (b) the outer surface of the fibers were also observed to be intact, however the connective tissue (arrows) between them was disrupted where enlarged gaps occurred; (c) large numbers of capillaries were found to be disrupted at 0 hrs, but rarely seen in tissues sampled at later time points. An intact endothelial cell (right arrow) is seen in a disrupted capillary, as well as extravasated red blood cells (left arrow). Extravasated red blood cells are also seen between muscle fibers at 0 hrs in the upper images. Bar = 100 μ m.

Figure 7.

Simulated displacement and von Mises strains dynamically induced using a single 50 HIFU pulse in an isotropic, linear elastic solid ($E = 27 \text{ kPa}$, $\nu = 0.499$). (upper left) Axial displacement (directed away from the HIFU piston) at the focal point. The cessation of the pulse is at 50 ms (indicated by the dashed vertical line). (upper right) The axial displacement field during steady-state sonication shows the displacement concentrated around the focal zone (a & b), while after cessation (c & d) of the sonication shear waves are released into the adjacent tissue outside the focal zone. (bottom) The lateral extent of the displacement (left) and von Mises strain (right) fields during the sonication ($< 50 \text{ ms}$) and after cessation of the sonication ($> 50 \text{ ms}$).

Figure 8.

Experimental displacement data acquired in mouse skeletal muscle *in situ*. (bottom) Mean focal point displacement through time immediately after cessation of a 50 ms HIFU pulse at three different power levels: 30 W (n = 6), 40 W (n = 4) & 50 W (n = 6). Sham pulses (0 W, n = 5) indicate the noise associated with data acquisition. The relaxation time back to the unperturbed state (0 μm) is 6–8 ms. (inset) Peak focal point displacement generated by a 50 ms HIFU pulse as a function of power (30, 40 & 50 W). Error bars (SE) represent the variability among different measurements.

Table 1

Characteristics of the different fluorophores used for the study

fluorophore	Excitation (λ)	Emission (λ)	size	administration
lectin (green)	488	515	100 kDa	systemic
BSA (green)	497	520	66 kDa	local
nanospheres (red)	580	605	100 nm	systemic/local
nanospheres (red)	580	605	200 nm	systemic