Focused Ultrasound Foundation Cover Letter

A) Proposal Title: Microbubbles-enhanced FUS for more efficient therapy for uterine fibroids.

B) Names, positions, addresses, institutional affiliations and contact information for all investigators, including the Principal Investigator and all Co-Investigators:

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C) Abstract: The goal of the research project will be to incorporate microbubbles in conjunction with FUS to decrease the time of FUS therapy of uterine fibroids. We believe that we can reduce the treatment times significantly while at the same time, reduce the mechanical energy required for tissue ablation. In addition, the use of microbubbles should provide for more precise tissue ablation of the fibroid such that viable tissue along the fibroid margins will be spared.

D) Amount of Funding Requested: $102,324

I. Initial Studies to Characterize Transducer Beam Pattern:

A) Physical Studies to Determine Focus: Initial studies were conducted to determine the focus and depth of penetration of the HIFU beam. Figure 1 below demonstrates the insonation patter of the parabolic transducer graciously provided by Mr. Ken Coffey of Artisonweb (Enola, Okla.).

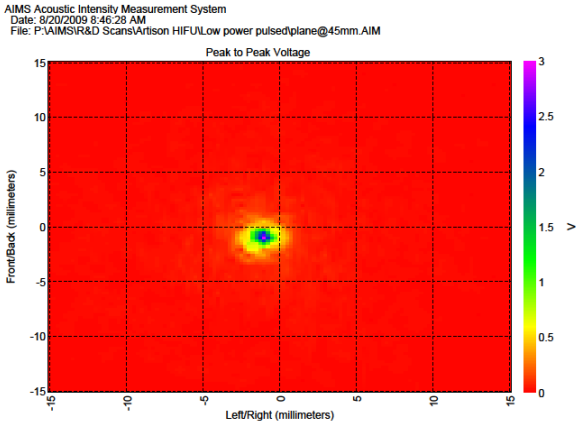


Figure 1. Plane Section of Insonation beam at 3.4 MHz.

A second cross-sectional image of the beam patter is demonstrated in Figure 2 below. The Focus of the beam patterned was measured at approximately 5 cm from the source.

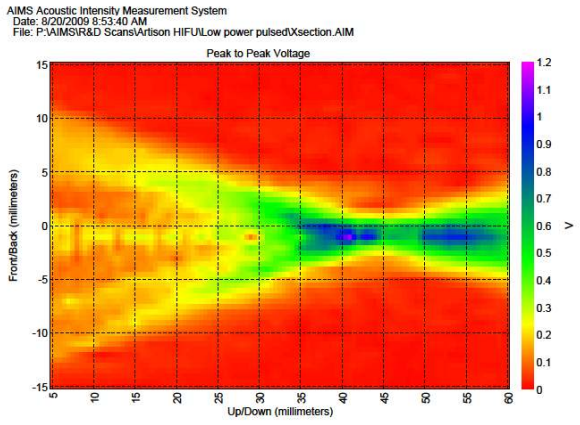
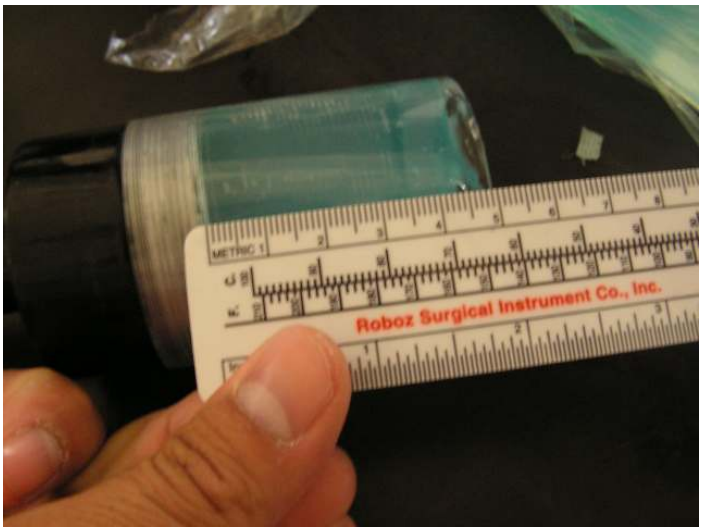


Figure 2. Cross-sectional beam pattern.



A 4 cm gel pad was then constructed (see Figure 3 below) followed by demonstration of initial HIFU insonation through chicken breast (see Figure 4). Note that the lesion in Figure 4 measures approximately 3 mm in the chicken tissue as opposed to the 1 mm beam theoretically proposed. This was due to the insonation being conducted over a prolonged period of time (approximately 2 minutes) at 500 W/cm^2 in order to visualize a "cooked" piece of tissue. Thus, the heating over a 2 minute period reflected cooking of the tissue in a radial direction, thereby the visually showing a larger radius than expected.

Figure 3. gel pad standoff constructed for the HIFU transducer.

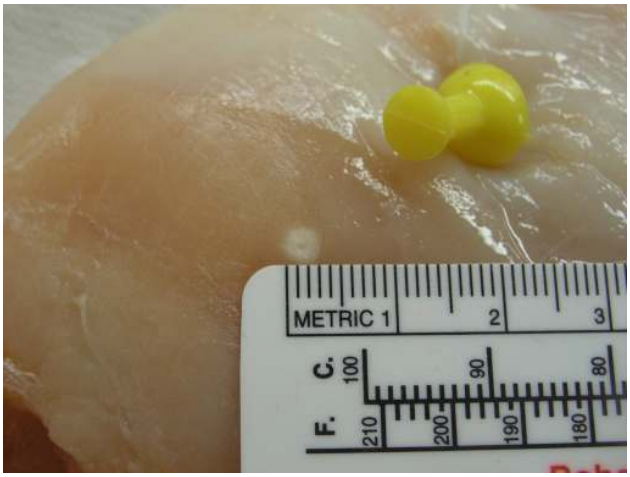


Figure 4 demonstration of tissue destruction with Artison HIFU transducer.

II. In Vitro Validation Studies for Closed Caspase and HSP-70 in Mammalian cells.

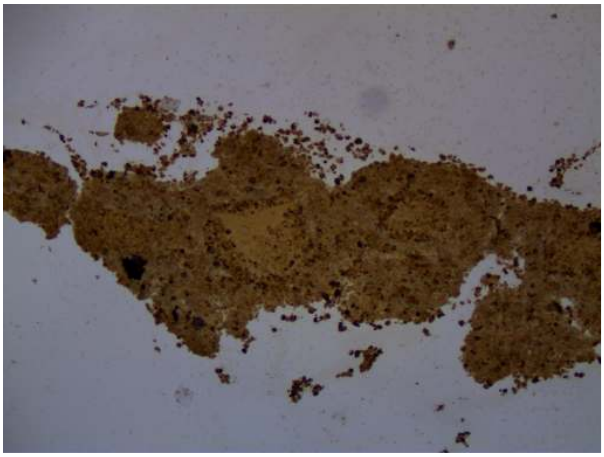
A) Closed Caspase Immunohistochemistry Assay: The samples were first processed and embedded in paraffin. Routine hematoxylin and eosin (H&E) stains were performed on three micron sections of tissue cut from the formalin fixed, paraffin embedded (FFPE) blocks. Immunohistochemistry (IHC) was performed using; a) Cleaved Caspase 3 (Cell Signaling #9662) rabbit polyclonal antibody diluted 1:60; and b) HSP70 (HSP 72) StressGen Assay Designs mouse monoclonal antibody (C92F3A-5) diluted 1:100.

Tissue sections were stained on a Discovery XT Automated Immunostainer (Ventana Medical Systems, Inc, Tucson, Arizona). All steps were performed on this instrument using VMSI validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate-EDTA buffer), primary antibody staining, detection and amplification using a a biotinylated-streptavidin-HRP and DAB (Diaminobenzidine) system and hematoxylin counterstaining. Cleaved caspase 3 was detected using a goat Anti-Rabbit secondary antibody and an UltraMap DAB detection kit (VMSI).

Following staining on the instrument, slides were dehydrated through graded alcohols to xylene and coverslipped with mounting medium (Richard Allan #4112).

Images were captured using an Olympus BX50 and Spot (Model 2.3.0) camera or Paxcam 3 camera with PAX-it Digital Image Management & Image Analysis. Images were standardized for light intensity. No automated analysis of the data was performed.

Figure 5 below is a demonstration of the presence of cleaved caspase which is an indicator of apoptosis. The image on the left demonstrates a control ensemble of cells where no apoptosis is present. The image on the right demonstrate the region of cells stained positive for cleaved caspase.



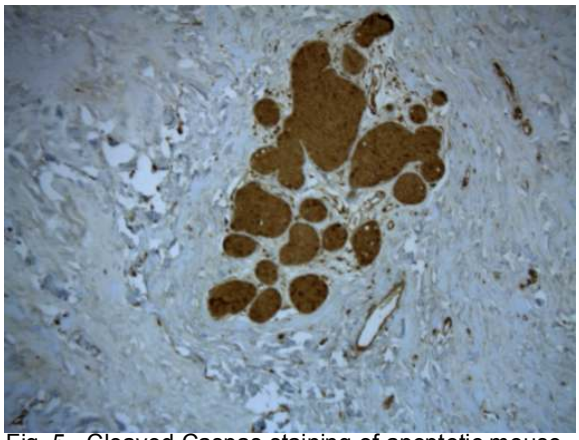


Fig. 5. Cleaved Caspas staining of apoptotic mouse skin tumor cells.

Figure 5. Cleaved Caspase Validation assay in mouse basal skin cell tumor. Left: Control. Right: Cleaved Caspase Positive.

B) HSP-70 Assay Upregulation: Validation assays have been performed in normal prostate and prostate glands with HSP-70 upregulation. These are seen in Figure 7 below:

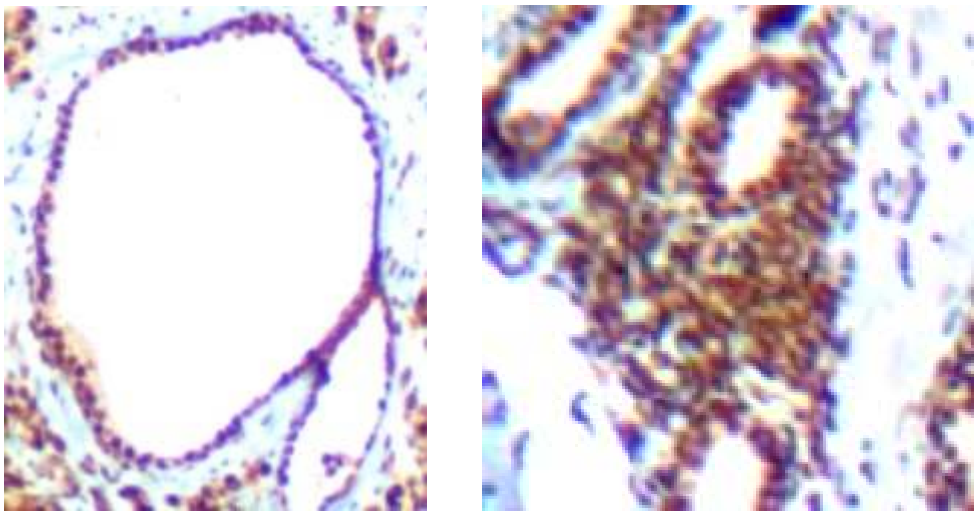


Figure 7. Left: normal gland tissue stained with HSP-70. Right: Normal prostate gland stained with HSP-70. Note different staining patterns. Note significant upregulation of heat shock protein on the right.

III. In Vivo Studies in New Zealand White Rabbits.

A) IACUC Approval: IACUC approval was obtained on July 10, 2009 by the University of Arizona IACUC. A copy of the final accepted protocol is provided in Appendix 1. A copy of the approval is provided in Appendix 2.

B) Materials and Methods: Rabbit HIFU Experiment Materials and Methods: Three to 5 kg New Zealand White rabbits were obtained through the University Animal Care Center and quarantined for five (5) to seven (7) days on unrestricted food and water diets. Rabbits were placed into containers by picking it up from the scruff of the neck ands supporting its hind legs to prevent self-induced spinal injury to the rabbit. Once in the surgical room they were placed into a cage and connected to an anesthesia up vaporizer. Rabbits were initially administered 5% isofluorane until sufficiently anesthetized. Approximately 10-15 minutes of anesthesia was utilized to ensure that the rabbit had entered an induced state prior to processing the insonation areas. The rabbit is taken out of the cage and placed into a gas cone which continued to deliver the 1% -2% maintenance dose of isoflurane. The insonation was performed on both of the hind legs in the thigh muscle to prevent interference with bone or excessive fat, and to better simulate insonation through muscle tissue. Clippers without a cover were used to get rid of both the top and under coat of the rabbit's fur. The shaved region was then wiped with isopropanol followed by wetting with sterile water.

The rabbits were then transferred to a second operating table and maintained on the isoflurane. An injection of Carprofen (4 mg/kg, (SC) was then administered prior to injection. In order to monitor the rabbit's vitals, the heart monitor was connected via a clip to an ear vein. When the vitals were stable, insonation commenced

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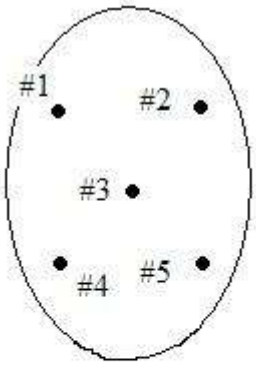
The insonation spots were chosen as a group and marked with a marking pen before any insonations began (see Figure 6 for general marking pattern). The transducer was centered over each spot, and for each insonation a temperature probe was (initially) placed 10-15 mm away from the center of the transducer to ensure accurate temperature readings during the insonation. After the first insonation the temperature probe

was placed further away in order to not harm the rabbit from the probe itself being heated by the high intensity focused ultrasound, and was later removed altogether due to difficulties with the temperature probes' durability while being inserted into the rabbit's thigh muscle. A 4 cm standoff gel pad was utilized after initial experiments demonstrated a focal point approximately 5 cm from the source. As such, it was estimated the region of focus would be approximately 1 cm into the tissue.

For the first rabbit, the power was initially set to 500 W/cm^2 varying for 15 to 30 seconds. After the initial 30 second trial on spot #1, we reduced the time and power to better reflect the types of procedures we aim to perform in the future, and brought the power down to 250 W/cm^2 for 15 seconds. For the first rabbit, both sides considered to act as controls. Aside from the mishap with the temperature probe for the insonation on the proximal medial spot on the rabbit's left leg (spot #1), spot #5 on both sides of the leg were performed with the temperature probe .7cm away from the center of the insonation point. For the second rabbit's insonation period, no temperatures were taken, and the power was set at 1.5W/cm^2 for ten seconds a spot. The rabbit's left thigh was, once again, simply a control side. Before insonating the right thigh, microbubbles (10-20 microliters per kg of animal's body weight, intravenously), made from dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidic acid, and dipalmitoylphosphatidylethanolamine-polyethylene glycol 2000 (or 5000) at a concentration of less than 2 mg/mL. The diluent is comprised of propyleneglycol, normal saline (or phosphate buffered saline), and optionally glycerol.

Following one week of recovery to allow the rabbit to heal from the procedure before harvesting the tissue. First, the rabbit is moved back to the surgical room as above, however the rabbit was not anesthetized by 5% isoflurane, but instead is given .5 – 1 cc/kg (IM) of "Rabbit Mix," (Xylazine HCI 160 mg (8 cc of 20mg/cc), Ketamine HCI 500 mg (5cc of 100 mg/cc), Acepromazine maleate 20 mg (10 mg/cc)). Once the rabbit had been induced, and the vitals stabilized, the Beuthanasia at 1cc/10 and is injected intravenously. Once positive that the drug has worked, we make sure we are able to see each insonation spot made by the marking pen a week earlier. Before cutting any tissue, a tissue dye was injected directly into the tissue to act as a marking dye for when the skin, and thus initial marks, are moved. Initially the skin is cut through first, in a wide arc that encompasses all of the insonation spots. Once cut and peeled back, the fascia is removed around the whole area. With muscle being the majority of tissue which we are cutting through, we are able to make cleaner, more precise cuts around the insonation spots. When harvesting the muscle tissue we first cut around the insonation spot in a square shape, 1/8 to ¼ inches from the outside of the marking dye. This gives us a little bit of room for error, and ensures our procedures for removing the tissue doesn't interfere with the cells in the insonated area. Once the tissue is removed from the rabbit, it is placed into a centrifuge tube filled with 10% formalin for 24 hours. The tissue samples are then placed in centrifuge tubes filled with 70% ethanol at 0°C and stored until processed by Dr. Ray Nagle's lab.



Rabbit Thigh Spot Layout:
#1: Proximal Medial
#2: Proximal Lateral
#3: Central
#4: Distal Medial
#5: Distal Lateral

Figure 6. Diagram of insonation pattern in Rabbits.

III In vivo studies in New Zealand White Rabbits

To date, two (2) rabbits have been studied, the first as a test control and the second as experimental and control in conjunction with microbubbles. A total of 18 samples have been excised, fixed and analyzed to date. Table 1 below notes the samples:

Sample	**Power Output**	**Insonation Time**	**N**
Control	500 W/cm^2	15 seconds	4
Control	250 W/cm^2	15 seconds	4
Microbubbles 20 microliters/kg	500 W/cm^2	15 seconds	5
Control	500 W/cm^2	15 seconds	5

A. Immunohistochemistry and Histology: IHC slides have been reviewed by Professor Raymond Nagle, M.D., Ph.D. in the Department of Pathology at the Arizona Health Sciences University. To date, both control HIFU samples and Microbubble + HIFU samples have exhibited clear coagulative necrosis with clear demarcations. Figure 7 below are slides of both control samples (500 W/cm^2 x 15 seconds) and Figure 8 experimental microbubble +HIFU (500 W/cm^2 x 15 seconds).

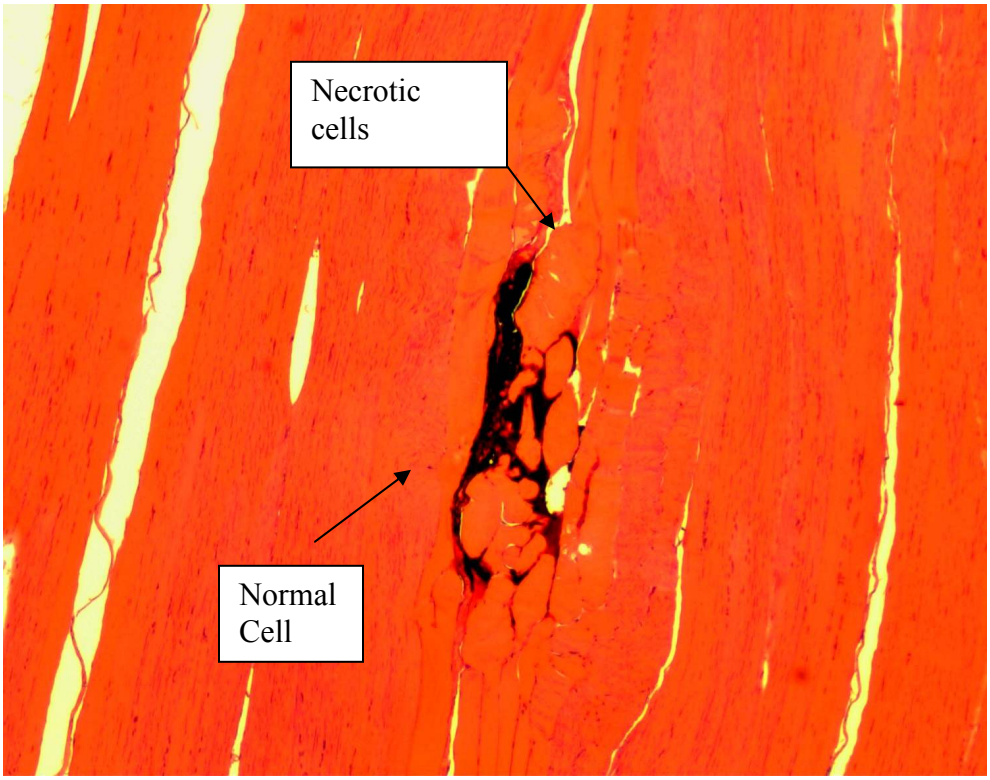


Figure 7. Control tissue. 500 W/cm^2 (No microbubbles)

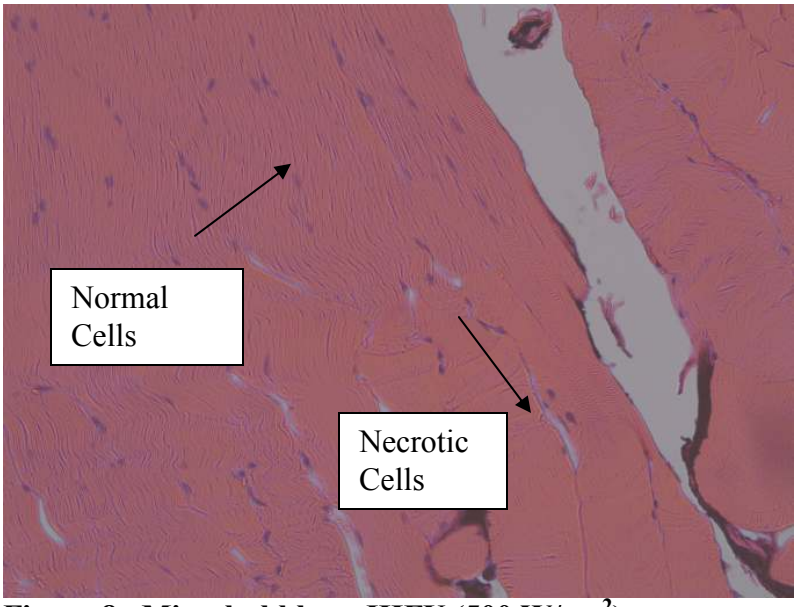


Figure 8. Microbubbles + HIFU (500 W/cm^2)

IV. Plans for remaining six (6) Months: Having validated the assays and identified the pathology using both control HIFU and microbubbles + HIFU. We now intend to complete the remaining profile of lesions as explained in the original protocol. Our goal is to obtain significantly more lesions so as to obtain better statistical significance levels with our study.

Appendix 1. IACUC Approval

Note: See pdf. file enclosed