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) Immunohistochemistry studies utilizing cleaved caspase and HSP-70 biomarkers: As noted in our previous report dated February 9, 2010, we had previously demonstrated that we can identify biomarkers for both apoptosis (cleaved caspase) and inflammatory repair responses (HSP-70). As an indicator, the Figures 1 and 2 below demonstrate both positive cleaved caspase and HSP-70 upregulation in mouse tumor models:

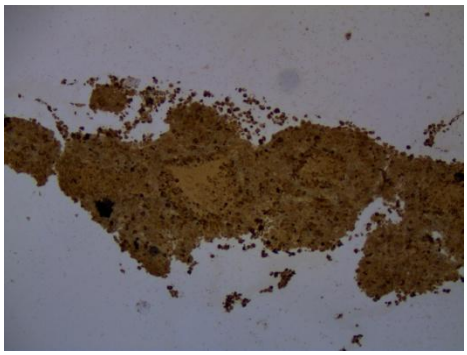
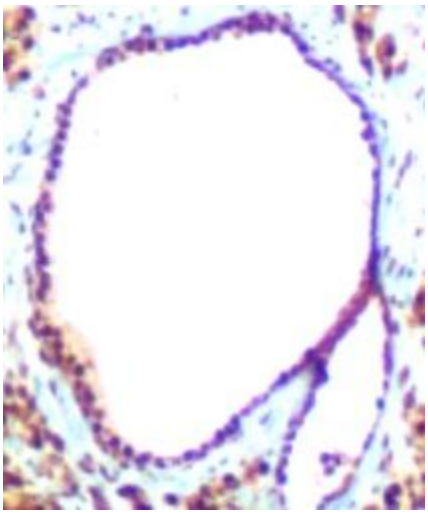


Fig. 5. Cleaved Caspas staining of apoptotic mouse skin tumor cells.

Figure 1. 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:



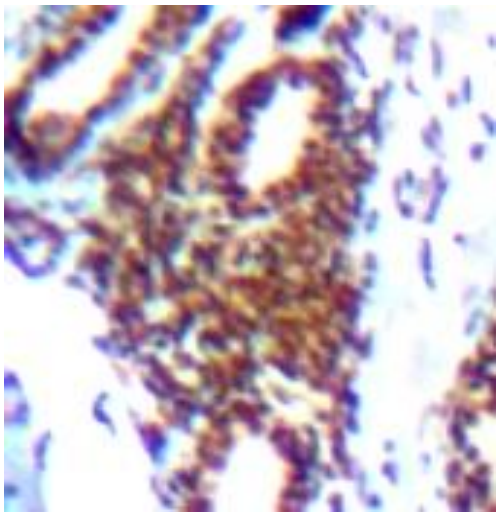


Figure 2. Left: normal gland tissue stained with HSP-70. Right: Normal prostate gland stained with HSP-70. Note different staining patterns. Note significant upregulation of heat shock protein on the right.

C) Cleaved Caspase and HSP-70 assays in rabbit HIFU ablation model: We have commenced assay experiments to identify evidence for apoptotic lesions or inflammatory-based upregulation of HSP-70 in the treated rabbit model.

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 and 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 remove the top and under coat of the rabbit's fur. The shaved region was then wiped with isopropanol followed by wetting with sterile water to prevent topical irritation.

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 1the 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 point on the rabbit thigh muscle. A 3.5 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 1.5 cm into the tissue.

A total of seven rabbits have been used to date. For the most recent rabbits, #6, the power was initially set to 500 W/cm^2 varying for 30 seconds. For rabbit #7, the power was initially set to 1 KW/cm2 with insonation for 30 seconds. A total of 9 insonations (18 total per rabbit) were conducted on each thigh muscle. 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.

Table 1 below is a summary of the latest experiments:

Rabbit #	Power Output at source	Predicted Power 1.5 cm in tissue
#6	500 KW/cm^2	273 W/cm^2
#7	1 KW/cm^2	546 W/cm^2

Table 1. Power output at source and predicted insonation power in tissue (est.)

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.

Tissue was then fixed in 10% neutral buffered formalin for 24 hrs, processed and embedded in paraffin. Tissue sections were cut onto a positively charged sides at 3um, baked for 1 hr for one hour at 65oC. Immunohistochemistry was done using a rabbit affinity purified antibody against Cleaved Caspase-3 (Cell Signaling technology) and a mouse affinity purified antibody against HSP-70 (Assay Designs Stressgen). The antibodies were diluted to 1:60 and 1:100 and stained on a Discovery XT Automated Immunostainer (Ventana Medical Systems) using DAB detection and counter stained with hematoxylin.

Results: We first identified regions of cellular abnormality using optical microscopy (note: tissue were not always necrotic, but abnormal as determined by disturbances in actin-myosin register and increases in interstitial space. Occasional necrosis was observed). Samples were then identified and quantified.

Table 2 below indicates the number of lesions positively identified:

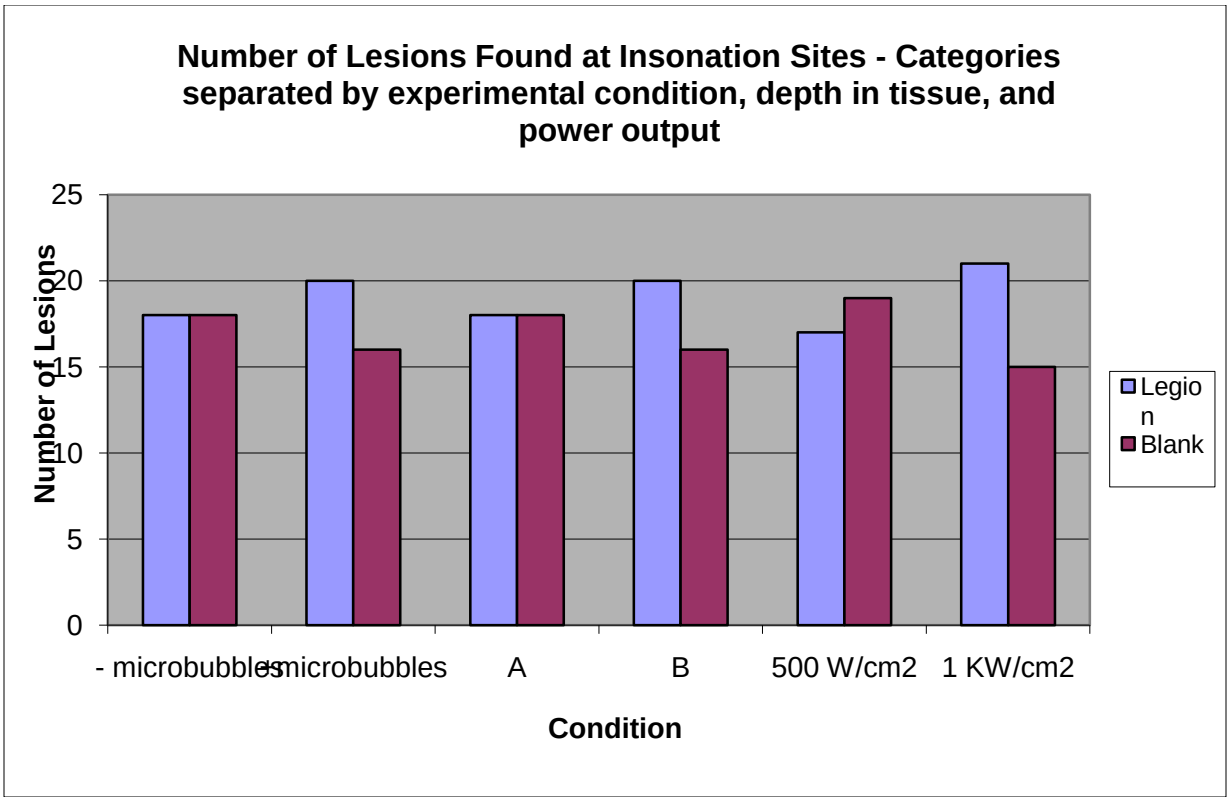

Table 2 Percent of potential lesions found by optical microscopy. Note: Cellular abnormalities (NOT NECROSIS) were most often identified.

All paraffin fixed samples subject to H&E (hematoxylin and eosin) staining were observed at up to 40X magnification on an optical microscope. Samples were observed for increases in interstitial space (indication of muscle tears secondary to lesions), abnormal actin-myosin registers, or muscle fiber contraction (an indication of tissue destruction or post-sacrifice spasms).

Figures 3 and 4 below are images of a normal (Figure 3) and abnormal (Figure 4) tissue.

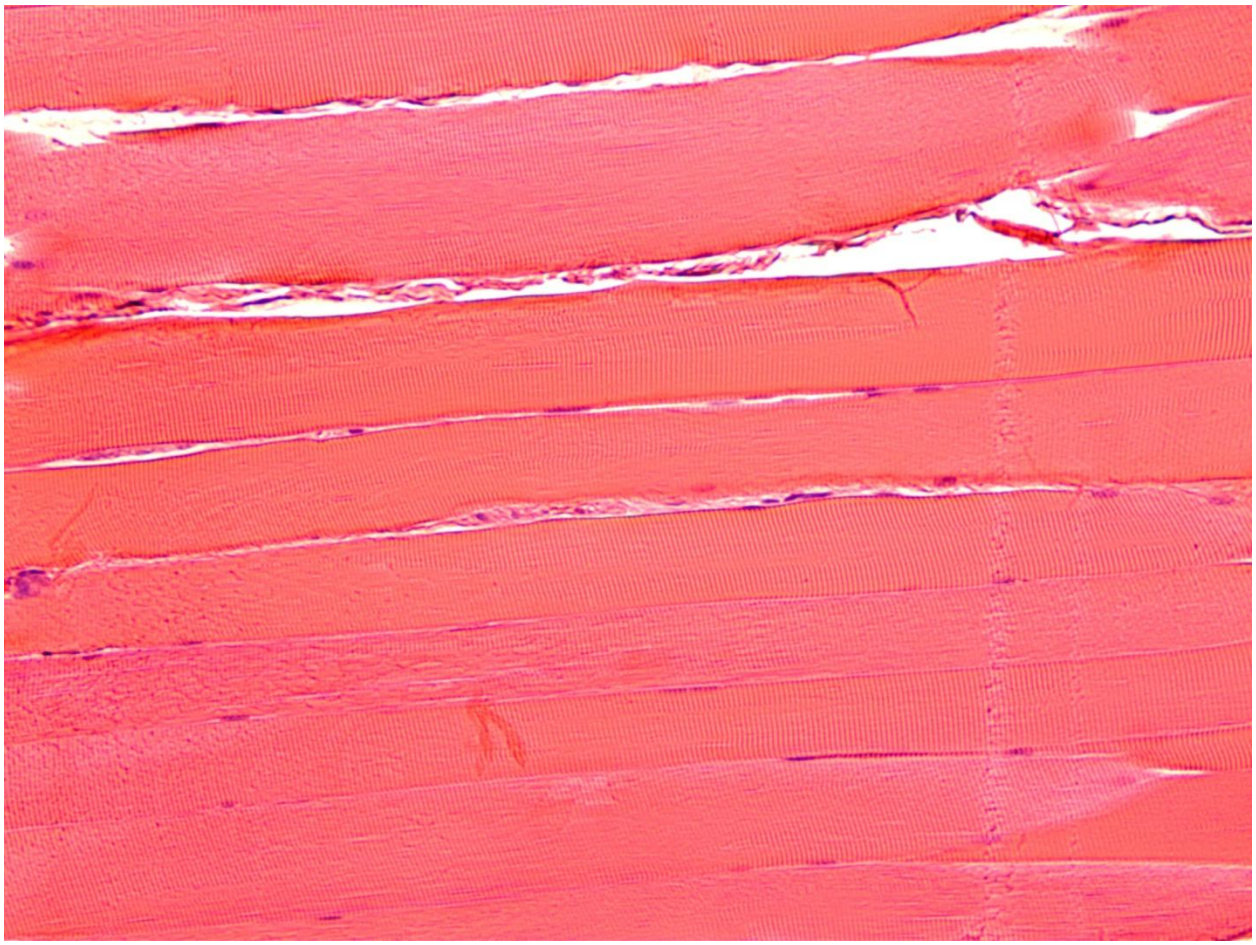

Figure 3. Normal thigh muscle tissue (image L2B). Note regular actin-myosin registers.

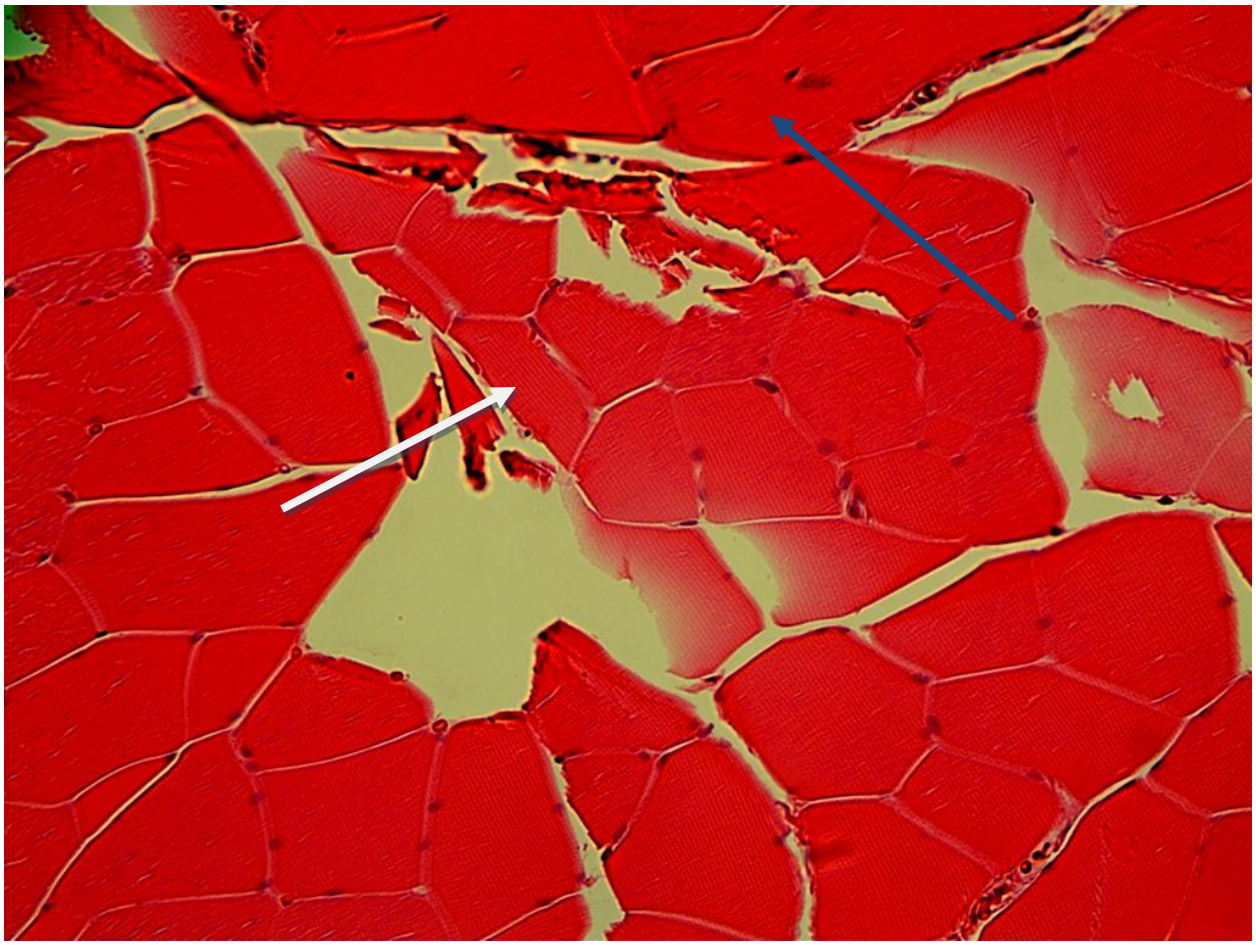

Figure 4. Fixed rabbit thigh muscle tissue outlining abnormal (white) vs. normal (blue) cellular tissue. Note the decrease in actin-myosin register. (image 7B)

The appropriate paraffin-fixed samples where lesions were identified were then assayed for apoptosis and HSP-70 upregulation. The assays are noted below in Figures 5 and 6.

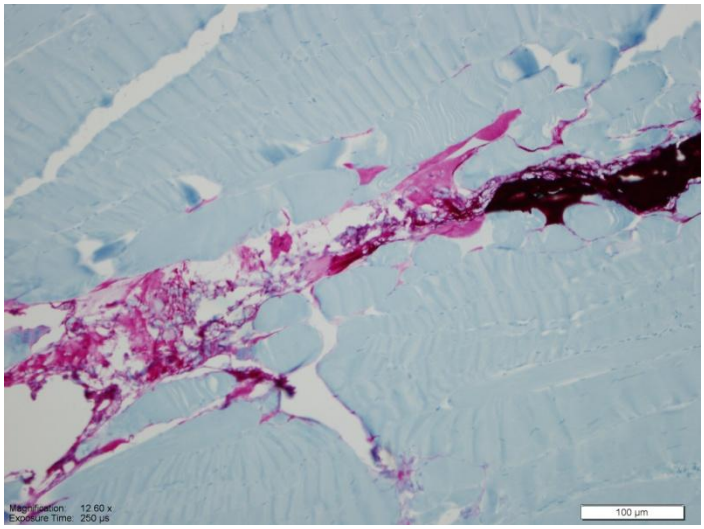

Figure 5 Image of Assay for HSP-70 in rabbit thigh muscle tissue. Note stain identifying region of cellular abmormality. Note no evidence for positive staining or upregulation of HSP-70.

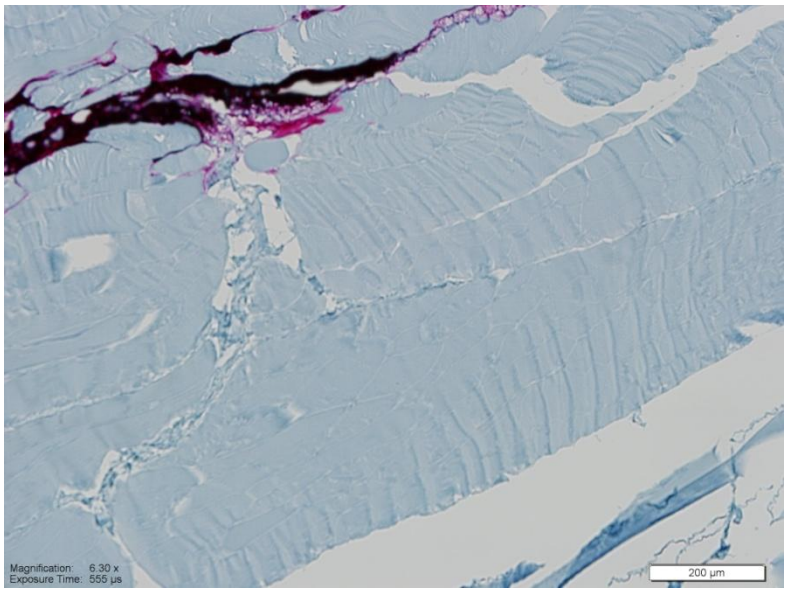

Figure 6. Tissue Image of Cleaved Caspase stained tissue in rabbit thigh muscle. Note stain identifying region of cellular abnormality. No evidence for positive staining or indication of Cleaved Caspase activity.

Results: As noted in the Figures, there was no evidence for apoptosis or HSP-70 upregulation in the peri-abated space.

Conclusion and Future Directions: We have been able to identify areas of abnormal cell distribution after tissue ablation with HIFU. Somewhat surprisingly, the peri-ablated space appears to be devoid of compromised tissue. Initially, there appears to be little difference between HIFU experiments with and without microbubbles. We are now assessing the level of sensitivity of the cleaved caspase and HSP-70 assays in order to determine the level of sensitivity for identifying lesions. In addition, recall that the rabbits are allowed to recover for seven days following HIFU. Another possible reason for little evidence of upregulation could be the timeframe for recovery. As such, we will conduct two additional experiments whereby our period of recovery will be 48 hours vs. seven days (168 hours). We will conduct these experiments utilizing the same power settings and insonation periods as previously utilized.