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The completed phantom investigation described in the half-term report was described in a manuscript; received a positive review and is currently under revision at the Journal of Applied Clinical Medical Physics. Briefly, we investigated the effect of normal incidence reflections from air, acrylic (modeling bone), and rubber on treatment location, temperature elevation, and heating patterns by performing ultrasound exposures in a tissue mimicking phantom and in *ex vivo* porcine tissue using a clinical MR-HIFU platform. The results demonstrated a shift in treatment location toward the distal interface, when targeted closer than 2 cm from the interface, especially for acrylic. Our study demonstrated that the ultrasound wave reflections from a distal air interface had less effect than the acrylic interface (modeling bone) on the heating pattern and focal location. This study provided useful information to better understand the limitations and safety concerns of performing MR-HIFU treatments with commercial clinical equipment.

Knowledge of the potential displacement of aimed treatment cells informed the next stage of our work, which involves live rabbits. An initial investigation was performed testing a variety of parameters to create internal tattoo marks as described in our proposal. A pilot sample of rabbits was initially used to determine if continuous wave HIFU would be able to make a conspicuous mark or 'tatoo'. Some limited success was seen here. The targeted regions of “cooked” tissue were visible on MRI, and on ultrasound, which is unsurprising. However the ultrasound also showed general edema that complicated the finding. In only one pilot case was the mark generated by continuous-wave FUS visible on CT.

This led us to investigate a combination of pulsed and continuous wave focused ultrasound. Theoretically a pulsed wave can cause damage to blood vessels and other tissues, resulting in a small amount of hemorrhage as well as mechanical tissue damage and edema (these are point sonications, not volumetric). This hemorrhaged blood, edema, and damaged tissue can then be essentially “burnt” in place using continuous wave ultrasound after some minimal delay. We investigated a variety of powers (between 50 and 140 watts) durations (sonications can last between 5 seconds and 2 minutes) delays (time between sonications varies from 15 seconds to 2 minutes) and combinations (CW followed by pulsed followed by CW, or pulsed followed by two different CW).

We now have an optimized HIFU tattooing protocol that creates conspicuous tattoo marks in *in vivo* tissue; the protocol includes a 100 second pulsed sequence followed by a 40 second 80 watt continuous wave sonication. This produces marks that are visible on MRI, and CT. Marks have also been seen on ultrasonography, but not with the same consistency. This work is being prepared as a manuscript to submit to Radiology, and a copy of that manuscript will be forwarded to you within the next few weeks.

Our protocol for marking/tatooing *in vivo* animal muscle tissues is summarized as follows: We used white New Zealand rabbits and an integrated clinical MRgFUS platform (Philips Sonalleve 1.5T Achieva). Before and after the HIFU tattooing procedure a 3D T2-weighted Turbo Spin Echo and fluid attenuated inversion recovery (FLAIR) sequence were acquired. Following the sonication procedure, CT scanning and ultrasonography were performed. Marks were excised and the tissue samples scanned digitally. On imaging, marks were located and confirmed by a radiologist with 15 years experience. Corresponding marks between CT and MRI were determined. Hounsfield units are measured in a tattooed region and compared to the Hounsfield units in a healthy tissue background ROI. For the MRI scans, signal intensity was measured in the tattooed region and normalized relative to the signal intensity of water from the degassed water bath in the same image. This was compared to the intensity in the healthy muscle tissue normalized to the intensity of water. A two tailed t-test was performed to determine if the populations of tattooed vs. healthy tissue were statistically significantly different, treating each individual tattoo as an independent data point, rather than averaging over all tattoos for a given rabbit. MRgFUS-generated tattoos are conspicuous on FLAIR MRI, standard CT imaging and clinical ultrasonography. In addition they are palpable and visually conspicuous upon excision.

The MR images acquired using a FLAIR sequence (often utilized for localization of brain edema) show the tattoos to be circumscribed areas of intensity, often with a brighter rim. On CT the lesions presented as more radiolucent than the surrounding healthy tissue. Both of these results are consistent with the firm nature of the tattoos on palpation during excision. The white color is consistent with muscle protein denaturation, and the fluid edema is most conspicuous on the edges of the tattoo. Due to the nature of ultrasound as an interactive, real-time modality, it is difficult to demonstrate correspondence robustly between MRI or CT with US in this setting. However, marks consistent with those observed in the other modalities are seen on ultrasound. The presentation on US is consistent with the edema-like tattoos observed on MR, CT and at excision.

In conclusion, we have completed one phase of the project and a manuscript regarding that work is under revision. A second manuscript is nearly completed regarding the heart of the project and will be provided to you shortly; we developed a method for marking *in vivo* animal muscle tissues. MRgFUS-generated tattoos are conspicuous visually, on FLAIR MRI, standard CT imaging, and clinical ultrasonography. The work performed to date involved sacrificing the rabbits after the HIFU tattooing procedure. We have submitted a protocol to perform a survival study in order to investigate tattoo durability, and hope to accomplish this quickly with our remaining funds.