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TITLE: Ultrasound to diagnose and treat heterotopic ossification

PRINCIPAL INVESTIGATOR: Julianna Simon

CONTRACTING ORGANIZATION: The Pennsylvania State University, University Park, PA

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14. ABSTRACT

The purpose of the project is to investigate Doppler ultrasound imaging and therapeutic ultrasound histotripsy for the diagnosis and treatment of acquired heterotopic ossification (HO). Thus far, we have imaged four rounds of ossified and control stem cells with color Doppler ultrasound and have found that imaging at 5 MHz with 12 cycles repeated at 3000 Hz and acoustic pressures of 2 MPa positive and 1 MPa negative produces the most twinkling. We continue to evaluate which metrics of mineralization best match Doppler ultrasound power and/or variance; a manuscript of these results is in preparation. We have also shown that therapeutic ultrasound histotripsy at 1.07 MHz with acoustic pressures of 12 MPa positive and 6 MPa negative successfully fractionates lab-grown calcium phosphate crystals less than 1 mm in size with minimal damage to the surrounding tissue-mimicking phantom. We are currently refining these parameters in ossified stem cells embedded in tissue-mimicking phantoms. Animal studies for imaging HO and treating HO are poised to begin pending receipt of the backordered Matrigel, a basement membrane needed to induce HO in the chosen murine animal model. On the benchtop, we have shown very promising results in detecting and treating HO that we will use to finalize imaging and therapy parameters before translating to the murine animal model of HO in the second year of the project.

15. SUBJECT TERMS
Heterotopic ossification, Doppler ultrasound, twinkling artifact, therapeutic ultrasound, histotripsy, mineralization

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1. **INTRODUCTION:**

Acquired heterotopic ossification (HO), or the presence of bone in soft tissues where bone does not normally exist, is the most common form of HO and occurs in <64% of military blast injuries to an extremity. Yet HO cannot be detected by computed tomography until at least 2 months post injury and physicians wait more than a year for HO to mature before treating by surgical resection to reduce the risk of recurrence. In this project, we are (1) evaluating the color Doppler ultrasound twinkling artifact to detect early signs of HO in mineral-depositing cells and in a murine model of HO; and (2) evaluating ultrasound-guided therapeutic ultrasound histotripsy to treat early HO in mineralized cells embedded in a tissue-mimicking phantom and in a murine model of HO. The goal of this project is to provide proof-of-concept for the detection and disruption of HO with ultrasound.

2. **KEYWORDS:**

Heterotopic ossification, Doppler ultrasound, twinkling artifact, therapeutic ultrasound, histotripsy, mineralization, human bone-marrow derived stem cells, osteogenic media

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
    1. Evaluate Doppler imaging in ossified and normal human bone marrow-derived stem cells. Target completion: May 31, 2022. Percent completion: 75%
    2. Obtain all regulatory approvals for testing ultrasound imaging and therapy in a murine model of heterotopic ossification. Target completion: August 31, 2021. Completion date: November 6, 2020.
    3. Evaluate Doppler ultrasound to detect early mineralization in a murine model of heterotopic ossification. Target completion: June 30, 2022. Percent completion: 20%.
    4. Evaluate therapeutic ultrasound histotripsy to fractionate minerals and surrounding cells in normal and ossified human bone marrow-derived stem cells. Target completion: June 30, 2022. Percent completion: 25%.
    5. Evaluate therapeutic ultrasound histotripsy to fractionate minerals and surrounding cells in a murine model of heterotopic ossification. Target completion: December 31, 2022. Percent completion: 10%.

- **What was accomplished under these goals?**

    1. Evaluate Doppler imaging in ossified and normal human bone marrow-derived stem cells.
        - Four rounds of imaging ossified cells with the color Doppler ultrasound twinkling artifact have been completed, with cells being imaged every third day from Day 10 – Day 25. We are currently working to complete one last partial round, where we hope to observe more mineralization. Concurrently, we are evaluating which quantitative metrics of mineralization to correlate with quantified metrics of twinkling. Preliminary results were presented at the Acoustical Society of America conference in Seattle, WA.
        - In this goal, our specific objective is to determine the sensitivity of color Doppler ultrasound to mineralization. To achieve this objective, we have been culturing human bone marrow-derived stem cells on gelatin and collagen scaffolds in osteogenic or control media. Cells were imaged with Doppler ultrasound every third day from Day 10 – Day 25. Doppler imaging parameters including frequency, pulse length, pulse repetition frequency, and acoustic pressure were modified at each time point and sample to determine the parameters that provide the sensitive detection of mineralization. Doppler ultrasound in phase-quadrature data was bulk saved for 30 seconds for each sample and imaging parameter set. After ultrasound imaging, samples were stained with OsteoImage mineralization

assay, and microscopy images captured from three locations across the scaffold for quantification of area mineralized, percent area mineralized, and mean mineralization size. Quantification of mineralization was compared to the Doppler magnitude and variance for each set of imaging parameters on each sample.

- We have completed four rounds of cell imaging with a fifth partial round currently underway. Initial rounds of cells did not show mineralization, even when staining with OsteoImage mineralization assay. Troubleshooting revealed older gelatin and cells and suggested a maximum scaffold thickness beyond which cells would not mineralize. New cells and gelatin were purchased and placed on thinner scaffolds – but the same results were found with little to no mineralization. Additional troubleshooting prompted us to switch to a collagen scaffold rather than gelatin. The biggest change from the imaging perspective is that cells migrate into the collagen scaffold instead of remaining on the surface. The change in cell scaffold has produced more mineralization and we have one more round of cellular imaging underway where we hope to achieve even more mineralization for comparison. Doppler ultrasound results show that twinkling is most prevalent at 5 MHz, with 12 cycles repeated in 14 ensembles at 3000 Hz and peak acoustic pressures of approximately 2 MPa positive and 1 MPa negative. Of note, these acoustic parameters are the maximum that can be safely achieved with the Verasonics® research ultrasound system and are well within FDA limits of diagnostic ultrasound imaging. Qualitative results from the OsteoImage mineralization assay shows disperse and "clumped" mineralization in cell samples cultured in osteogenic media; control samples cultured in growth media also show some disperse mineralization, but the quantity is lower than that found in the cells cultured in osteogenic media **(Fig. 1b).** When results are quantified as percent area mineralized or mean mineralized area, correlations begin to emerge with Doppler magnitude or variance in some samples, but not others **(Fig. 1a).** Ongoing work seeks to understand why some samples show good correlation between mineralization and twinkling whereas others do not. A manuscript is currently in preparation as the results start to become finalized.

2. Obtain all regulatory approvals for testing ultrasound imaging and therapy in a murine model of heterotopic ossification.
    - IACUC approvals were obtained at the Pennsylvania State University on July 24, 2020. ACURO approval was obtained on November 6, 2020.

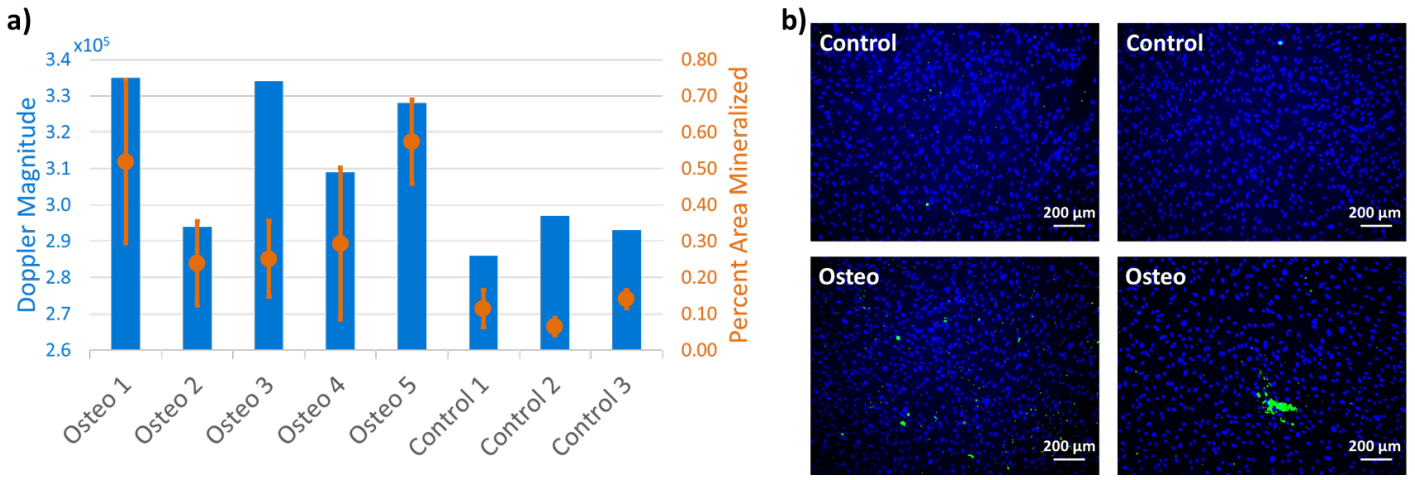

Fig. 1. a) Plot comparing Doppler magnitude (blue) to percent area mineralized (orange) for 5 samples cultured in osteogenic media and 3 samples cultured in normal growth media (controls) on Day 19. With the exception of sample Osteo 3, trends observed in Doppler magnitude agree with increasing percent area mineralized. **b)** OsteoImage mineralization assay results showing cell nuclei (blue) and minerals (green). Control samples show some mineralization, which is reflected in some Doppler signal in the graph in a). More mineralization appears in samples cultured in the osteogenic media with small minerals dispersed throughout the sample or larger "clumps" of minerals.

- The specific objectives were to obtain local IACUC (Pennsylvania State University) and ACURO approval for the animal studies.
- The attainment of these approvals was a major milestone on the approved SOW.

3. Evaluate Doppler ultrasound to detect early mineralization in a murine model of heterotopic ossification.
    - Students have completed all trainings (including hands-on training) necessary to conduct the proposed animal study. The experimental fixtures to hold the mice partially submerged in a water tank have been designed and tested in the lab.
    - The specific objective is to determine the earliest forms of HO that can be detected with Doppler ultrasound. To achieve this objective, we will induce HO in the gastrocnemius muscle of 15 mice and image with Doppler ultrasound every other day for two weeks using the technique we optimized when imaging ossified stem cells. A subset of mice will be euthanized after each imaging day for tissue collection and histological analysis. The Doppler ultrasound results will be correlated with histological results to determine the earliest signs of HO that can be detected with Doppler ultrasound.
    - We have not started collecting data for this study as we have not yet received the Matrigel we ordered in September. The Matrigel is a necessary component to hold the recombinant human bone morphogenic protein-4 (rhBMP-4) at the injection site to induce HO. Dates continue to shift back for expected delivery dates, with the current estimated delivery of April 1, 2022. We are exploring other basement membranes and talking with colleagues as we seek to overcome this supply chain delay. As soon as the Matrigel (or alternative) is received we are ready to start the study.

4. Evaluate therapeutic ultrasound histotripsy to fractionate minerals and surrounding cells in normal and ossified human bone marrow-derived stem cells.
    - We have conducted preliminary tests of therapeutic ultrasound histotripsy on lab-grown calcium phosphate crystals and are beginning another round of test on smaller crystals (all dimensions < 1 mm) as these are our target minerals to treat in HO. We have also designed a method for holding ossified stem cell samples in a tissue mimicking phantom and plan to begin collecting data the end of January.
    - The specific objective is to determine the histotripsy parameters that will fractionate small mineral depositions like those found in early forms of heterotopic ossification with minimal damage to the surrounding tissues. To achieve this objective, we began testing histotripsy parameters in lab-grown calcium phosphate crystals embedded in a tissue-mimicking phantom monitored with high-speed photography. We will then move onto fine-tuning histotripsy parameters in ossified stem cells embedded in a tissue-mimicking phantom.

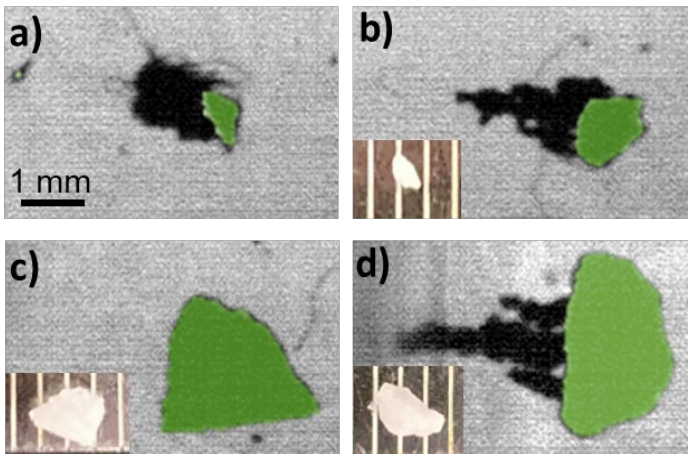

Fig. 2. High-speed photographs showing lab-grown calcium phosphate crystals (green) embedded in a polyacrylamide tissue-mimicking phantom. Insets show the crystal removed from the gel after treatment. **a)** A 0.75x1 mm crystal treated with focused ultrasound at p_+=12 MPa and p_-=6 MPa is completely fragmented. The phantom shows 0.5 mm^3 of damage from acoustic cavitation. **b)** A larger 1x1.5 mm crystal treated with the same parameters as in a). Upon removal, the crystal does not fully fragment but is smaller by about 40%. **c)** A 2x2.5 mm crystal treated with lower amplitude of p_+=7 MPa and p_-=4 MPa. No cavitation activity is visible and no fragmentation occurs. **d)** A 2x3 mm crystal treated with higher amplitudes of p_+=47 MPa and p_-=11 MPa. Little to no crystal fragmentation occurred accompanied by more phantom damage from cavitation.

- Preliminary studies in lab-grown crystals embedded in a polyacrylamide tissue-mimicking phantom shows we can successfully fractionate the smallest crystals (<1 mm diameter) with minimal damage to the surrounding tissues. In **Fig. 2a,** the smallest crystal with dimensions ≤1 mm showed complete fragmentation after treating for 5 minutes at 1.07 MHz with 20 cycles repeated at 200 Hz and pressure amplitudes of p_+=12 MPa (peak positive pressure) and p_-=6 MPa (peak negative pressure). Damage to the surrounding tissue from pre-crystal cavitation activity was approximately 0.5 mm^3. Larger crystals showed some "dusting" or partial fractionation on the edges (~40% reduction in size) with the same treatment parameters. Damage to the surrounding tissue-mimicking phantom remains similar for the same ultrasound parameter set of 0.6 mm^3 **(Fig. 2b)**. Repeated targeting at 3 different locations on the same crystal using the same acoustic parameters increased the fragmentation of the crystal to about 50% **(Fig. 3),** though caused more damage to the surrounding tissue-mimicking phantom totaling approximately 5.8 mm^3. When the acoustic pressure amplitude was reduced to p_+=7 MPa and p_-=4 MPa, no pre-crystal cavitation activity and minimal to no crystal fragmentation was observed **(Fig. 2c)**. Similarly, when the acoustic pressure amplitude was increased to p_+=47 MPa and p_-=11 MPa (same frequency and pulsing scheme) and a total treatment time of 70 seconds, minimal to no crystal fragmentation but more damage to the tissue-mimicking phantom from pre-crystal cavitation activity occurred (approximately 1.4 mm^3) **(Fig 2d)**. Our next step is to evaluate histotripsy parameters in additional sub-millimeter-sized, lab-grown crystals embedded in the tissue-mimicking phantom before evaluating histotripsy in ossified stem cells embedded in the tissue-mimicking phantom.

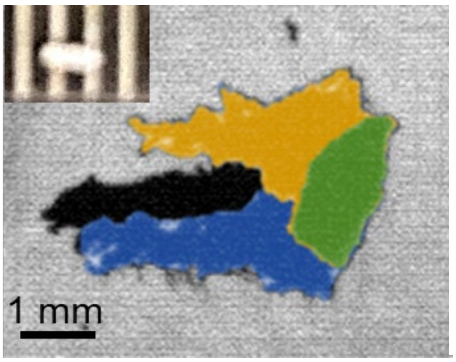

Fig. 3. High-speed photograph showing a 1x2 mm crystal (green) treated in 3 different locations with focused ultrasound at p_+=12 MPa and p_-=6 MPa. The crystal did not fully fragment, but was smaller by 50%. More acoustic cavitation-based damage was found in the gel from the multiple treatments totaling about 5.8 mm^3.

5. Evaluate therapeutic ultrasound histotripsy to fractionate minerals and surrounding cells in a murine model of heterotopic ossification.
    - Students have completed all trainings (including hands-on training) necessary to conduct the proposed animal study. The experimental fixtures to hold the mice partially submerged in a water tank have been designed and tested in the lab.
    - The specific objective is to determine whether therapeutic ultrasound histotripsy can fractionate minerals and surrounding cells in HO without increasing the risk of recurrence in mice. To achieve this objective, we will induce HO in the gastrocnemius muscle of 24 mice. Mice will randomly be assigned to early HO histotripsy treatment (n=8), mature HO histotripsy treatment (n=8), or sham (n=4 early HO, n=4 mature HO). Mice will survive for an additional 2 weeks after the histotripsy treatment before collecting tissues for histological analysis.
    - According to the SOW, this study is scheduled to start in the summer of 2022. Assuming we can obtain the Matrigel necessary to induce HO, we anticipate we are still on schedule to complete the study as planned.

- **What opportunities for training and professional development has the project provided?**
    - Lucas Ruge-Jones attended (but did not present at) the virtual conferences: Acoustics in Focus sponsored by the Acoustical Society of America (June 8-10, 2021), Symposium of

the International Society for Therapeutic Ultrasound (June 6-9, 2021), and the IEEE International Ultrasonics Symposium (September 11-16, 2021). These opportunities allowed Mr. Ruge-Jones to learn more about the state-of-the-art in diagnostic and therapeutic ultrasound, which is directly translatable to his role on this project.

- **How were the results disseminated to communities of interest?**
  - Results from the on-going Doppler imaging of normal and ossified cells study was presented by Dr. Simon on behalf of graduate student Lucas Ruge-Jones at the 181st Acoustical Society of America conference in Seattle, WA on November 30, 2021.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  1. Evaluate Doppler imaging in ossified and normal human bone marrow-derived stem cells.
     - We will finish up the last partial round of imaging ossified cells with Doppler ultrasound. We will determine which mineralization metric (e.g. mineralization size, quantity, total mineralization area, etc.) most closely matches the results from Doppler magnitude and/or variance calculations. Then, we will finish preparing the manuscript and submit for publication.
  2. Obtain all regulatory approvals for testing ultrasound imaging and therapy in a murine model of heterotopic ossification.
     - Goal is complete.
  3. Evaluate Doppler ultrasound to detect early mineralization in a murine model of heterotopic ossification.
     - We will continue to work to obtain the Matrigel basement membrane or similar product to initiate HO in the mice. Once the Matrigel is received, we will inject 15 mice with rhBMP-4 in Matrigel solution to induce HO. Then we will image mice every other day for two weeks to monitor for twinkling, and thus mineralization. Three mice will be euthanized on each imaging day for histological analysis tissues, and thus confirmation and quantification of mineralization. We will compare the Doppler ultrasound twinkling results to histological results to determine the earliest forms of HO that can be detected with Doppler ultrasound. These results will be presented at a conference (Acoustical Society of America, and/or IEEE International Ultrasonics Symposium) and a manuscript will be prepared and submitted for publication.
  4. Evaluate therapeutic ultrasound histotripsy to fractionate minerals and surrounding cells in normal and ossified human bone marrow-derived stem cells.
     - In this goal, we will continue exploring histotripsy parameters to achieve fractionation of minerals with minimal damage to the surrounding tissues in small (<1 mm), lab-grown calcium phosphate crystals to confirm our initial results. Then, we will test the same histotripsy parameters to treat ossified and control stem cells embedded in a tissue-mimicking phantom. At least 5 cell samples will be treated for each histotripsy parameters and for a range of cellular mineralization. At the end of treatment, gel samples will be photographed for quantification of damage to the tissue-mimicking phantom. Remnants of the phantom and scaffold will be collected for mineralization quantification via the OsteoImage mineralization assay and scanning electron microscopy. We will also collect the liquefied gel and stain with hematoxylin and eosin (H&E) to evaluate cellular debris and lipid membrane stain SPDiIC$_{18}$(3) pico green to detect nucleic acids. The histotripsy parameter set that disrupts the most minerals with the least damage to the tissue-mimicking phantom will be determined and used to complete goal 5. These results will be presented at a conference (Acoustical Society of America, International Symposium of Therapeutic Ultrasound and/or IEEE International Ultrasonics Symposium) and a manuscript will be prepared and submitted for publication.

5. Evaluate therapeutic ultrasound histotripsy to fractionate minerals and surrounding cells in a murine model of heterotopic ossification.
    - HO will be induced in twenty-four mice by injecting rhBMP-4 in Matrigel solution into a randomly-selected gastrocnemius muscle. The top histotripsy parameter set from the ossified cell study (goal 4) will be used to treat HO. Eight mice will be treated on Day 10, which has previously been shown to represent early HO in this animal model, 8 mice will be treated on Day 14 (mature HO), and 8 mice will be assigned to the sham group. Mice will be survived for two weeks after treatment before euthanasia. Bilateral gastrocnemius muscles will be collected for histological analysis with H&E, aniline blue and safranin O to evaluate for HO recurrence after histotripsy treatment. The results from this goal will be presented at a conference (Acoustical Society of America, International Symposium of Therapeutic Ultrasound and/or IEEE International Ultrasonics Symposium) and a manuscript will be prepared and submitted for publication.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
    - We have found that Doppler ultrasound imaging can be used to detect minerals deposited by cells. This result is novel in that it shows, for the first time, that Doppler ultrasound can be used to detect other forms of mineralization besides kidney stones – such as heterotopic ossification (HO). It also shows that ultrasound can detect sub-wavelength structures. This is very exciting as it opens up a whole new area of untapped potential in diagnostic ultrasound. We believe that small bubbles are present on the surface of these minerals and cause scattering that is detectable with Doppler ultrasound. On kidney stones, these bubbles have been found to be sensitive to changes in gas composition and pressure – which affects the twinkling artifact. This sensitivity of ultrasound to small gas pockets suggests, at some level, that ultrasound can be used to detect changes in metabolism. Should this prove true, this would be game-changing in using ultrasound for diagnosing a variety of diseases.
    - Additionally, the detection of ossified stem cells with Doppler ultrasound will greatly impact the diagnosis and allow for monitoring progression of HO. Instead of relying on patient-reported symptoms, or ionizing radiation-producing three-phase bone scintigraphy, computed tomography or plain film x-ray imaging, the use of Doppler ultrasound will provide a radiation-free, and thus safter, alternative to diagnose and track HO progression. This advancement in imaging will easily fit within the current standard-of-care for HO, where physicians wait for HO to mature before treatment. With ultrasound, more routine monitoring can be used to track HO maturity and thus enable earlier treatment and reduce sequelae associated with HO.
    - In addition to detection of HO, we have made advances in treating HO. Preliminary results show that we can use focused ultrasound histotripsy to treat calcium phosphate crystals grown in the lab. Should we find similar success in a larger number of lab-grown crystals and in a murine model of HO, we will have provided a non-invasive alternative to surgical resection to treat HO. The results will be especially profound if the use of focused ultrasound histotripsy does not cause recurrence of HO, allowing for immediate treatment of HO, reducing the societal cost of HO, and allowing military service personnel to return to active duty with minimal down time. Even if HO cannot be treated earlier with ultrasound, the benefits of a non-invasive surgery (i.e. lower risk of infection, less tissue trauma, etc.) greatly reduce the societal and military burden of HO.

- **What was the impact on other disciplines?**
    - Our finding that ultrasound can be used to detect and perhaps treat pathological mineralization could also help patients with gout, breast cancer with microcalcifications, or encrusted catheters/stents. Using Doppler ultrasound, we can improve detection of

minerals throughout the body and then use therapeutic ultrasound histotripsy to break up the minerals non-invasively. This approach will improve patient quality of life and will streamline healthcare for those suffering from pathological mineralization.

- The possibility that we can extend the results from detecting HO to cellular metabolism would have an even greater impact on a variety of disease. If we can extend the results from detecting ossified stem cells and HO to gases at the cellular level, we could detect earlier forms of a wide variety of diseases. This has the potential to change the role of ultrasound in medicine.

- **What was the impact on technology transfer?**
  - Nothing to Report

- **What was the impact on society beyond science and technology?**
  - Because of the portability and reduced costs of diagnostic and therapeutic ultrasound compared to other imaging and therapeutic modalities, we are reducing societal discrimination and potentially improving the quality of care for those in rural areas. This helps improve the socioeconomic and location health care discrimination by replacing expensive modalities only offered in large hospitals to cheaper and more portable ultrasonic technologies.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - In goal 1, cellular scaffolds were changed from gelatin to collagen to increase mineralization. Despite preliminary studies showing mineralization of human bone marrow-derived stem cells on the gelatin scaffolds, the first two rounds of cells for imaging showed little to no mineralization. Upon changing to the collagen scaffold, significantly more mineralization has been observed in cells cultured in osteogenic media.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - We have been unable to obtain Matrigel for inducing HO in the mice. The current estimated delivery date is April 1, 2022; however, this date continues to change since the order for Matrigel was placed in September. We are exploring other basement membrane formulations and are consulting with colleagues to see if we could borrow Matrigel until ours is delivered. The problem with other formulations is gelation time. Matrigel solidifies almost immediately, whereas many of the other formulations can take up to 30 minutes to solidify. Gelation time is critical for this animal model as the purpose of the Matrigel is to hold the rhBMP-4 in place to induce HO. If we are unable to obtain Matrigel in a reasonable time frame, we will consult with the agency Grants Officer to pursue other animal models of HO.

- **Changes that had a significant impact on expenditures**
  - One graduate student (Lisa Bernstein) was already slated to be supported by a teaching assistantship for Spring 2021 semester at the time the grant was awarded. This allowed us to re-budget expenditures for cellular supplies, which was needed to obtain the objectives in goal 1 as the initial cellular samples were not mineralizing as anticipated.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Nothing to Report.

- **Significant changes in use or care of human subjects**
  - Nothing to Report.
- **Significant changes in use or care of vertebrate animals.**
  - Nothing to Report.
- **Significant changes in use of biohazards and/or select agents**
  - Nothing to Report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
  - **Journal publications.**
    - Nothing to Report.
  - **Books or other non-periodical, one-time publications.**
    - Nothing to Report.
  - **Other publications, conference papers, and presentations.**
    - Ruge-Jones L, Bernstein L, Hayes D, Simon JC (2021). “Detection of cellular mineralization using the Doppler ultrasound twinkling artifact.” 181st Meeting of the Acoustical Society of America, Seattle, WA USA: November 29 – December 3, 2021.
- **Website(s) or other Internet site(s)**
  - Nothing to Report
- **Technologies or techniques**
  - Nothing to Report.
- **Inventions, patent applications, and/or licenses**
  - Nothing to Report.
- **Other Products**
  - Preliminary results from this project show the utility of Doppler ultrasound imaging and therapeutic ultrasound histotripsy to diagnose and treat HO. Doppler ultrasound imaging is being refined to enhance detection of HO and will shortly be tested in a murine model of HO. Similarly, therapeutic ultrasound histotripsy is being investigated for treatment of HO minerals and has shown early success in fractionating calcium phosphate crystals grown in the lab. These technologies have the potential of improving diagnosis and treatment of HO, which will reduce the patient and societal burden of HO and allow servicemembers to return to active duty.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Julianna Simon
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0002-0425-9939

Nearest person month worked:	1
Contribution to Project:	Dr. Simon maintains fiscal and administrative responsibility for the project. As such, she oversees her graduate student, Lucas Ruge-Jones, and meets regularly with her Co-investigator Dr. Daniel Hayes and his graduate student Lisa Bernstein. She also presented for Mr. Ruge-Jones at a recent Acoustical Society of America meeting.
Funding Support:	This Award

Name:	Daniel Hayes
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0001-9499-4111
Nearest person month worked:	1
Contribution to Project:	Dr. Hayes supervises his graduate student, Lisa Bernstein, and provide guidance on culturing ossified stem cells. He also participates in project meetings.
Funding Support:	This Award

Name:	Lucas Ruge-Jones
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Mr. Ruge-Jones has led the effort on Doppler imaging of normal and ossified stem cells, including collecting and processing the imaging data. He has also completed all trainings and experimental arrangement preparations for the upcoming animal imaging studies. Mr. Ruge-Jones also has started testing histotripsy fractionation of lab-grown crystals. Additionally, he prepared an abstract for presentation at the Fall 2021 Acoustical Society of America Conference.
Funding Support:	This award with tuition covered by the Graduate School at The Pennsylvania State University.

Name:	Lisa Bernstein
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	4
Contribution to Project:	Ms. Bernstein has led the effort in culturing the normal and ossified stem cells for Doppler imaging, including staining and collecting images of the distribution of minerals on the scaffolds for comparison with the ultrasound results. She has also completed all trainings and is prepared to help with animal care and histological analysis for the upcoming animal imaging studies.
Funding Support:	This award with tuition covered by the Graduate School at The Pennsylvania State University. Ms. Bernstein was also supported on a teaching assistantship for Spring 2021 semester.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  - **PI Simon:**

    - **PROJECTS FUNDED SINCE LAST REPORT**

Congressionally Directed Medical Research Programs (2/1/2021-1/31/2023)
Peer-Reviewed Medical Research Program Discovery Award requested Time: 0.25
Role: Simon Co-I; Project PI: D. Hayes months/year
Ultrasound responsive hydrogels for on-demand sustained drug delivery
The goal of this project is to demonstrate proof-of-concept focused ultrasound-responsive hydrogels to provide spatiotemporal release of BMP-2 for the directed repair of segmental bone defects. Aim 1: Determine the influence of Diels-Alder cross-linkage composition on the focused ultrasound response of hydrogels and associated release of BMP-2. Aim 2: Explore the influence of the focused ultrasound-mediated release of BMP-2 from hydrogels on bone regeneration.

    - **PROJECTS COMPLETED SINCE LAST REPORT**

Nothing to Report

  - **Co-I Hayes:**

    - **PROJECTS FUNDED SINCE LAST REPORT**

R25 EB027620-01A1 Hayes (Co-PI) 9/23/2021-03/31/2026 0.06 months
NIH NIBIB
Biomedical Engineering Design: Integrating Simulation, Clinical Immersion, and Regulatory Training
This project develops an instructional program for biomedical engineers focused on simulation, quantitation, design and regulation in the design of biomedical devices.

Aura Biosciences Hayes (PI) 03/15/2021 - 0.25 months
03/14/2022 Industrial Research Support
PKA Diagnostic Development Project
This project is focused on the optimization of new tools and techniques for determining the pharmacodynamics of a new particle based therapeutic in human clinical trials.

D01 W81XWH2110052 Hayes (PI) 2/1/2021-1/31/2023 0.25 month
Congressionally Directed Medical Research Programs
Ultrasound Responsive Hydrogels for "On-Demand" Sustained Drug Delivery
This proposal addresses the development of a next generation ultrasound controlled drug delivery system, composed of a reversibly Diels-Alder crosslinked polyethylene glycol (PEG) hydrogel delivering BMP.

- **PROJECTS COMPLETED SINCE LAST REPORT**

No number Hayes (Co-PI) 07/01/2019 – 06/30/2021 0.12 cal months
Grace Woodward collaborative grant
miRNA enhanced vasculogenesis for treatment of diabetic microvascular dysfunction
This project seeks to identify miRNA variations in non-healing lower limb diabetic wounds and examine the influence of miRNA replacements on the vasculogenic potential of EPCs.

D01 W81XWH1810115 Hayes (PI) 05/15/2018 - 11/14/2021 1.5 cal months
DOD / CDMRP Discovery Award
Magnetically Actuated Therapy for Bone Regeneration
This project explores RF modulated strategies providing spatiotemporal control of gene regulation in endogenously derived MSC to improve the repair of critical sized bone defects.

- **What other organizations were involved as partners?**
  - Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**
   - Nothing to Report

9. **APPENDICES:**

Abstract for the 181st Meeting of the Acoustical Society of America, Seattle, WA USA: November 29 – December 3, 2021.

Detection of cellular mineralization using the Doppler ultrasound twinkling artifact.

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Heterotopic ossification (HO), or the growth of bone in soft tissue where bone does not usually exist, can occur after any musculoskeletal trauma. While the exact cause of HO is unknown, it can take 2-3 months before HO can be detected on x-ray or computed tomography (CT). The color ultrasound Doppler twinkling artifact is a rapid color shift that was discovered when imaging kidney stones. Here, our objective is to determine whether twinkling can be used to detect the earliest signs of cellular mineralization in HO. A Vantage-128 research ultrasound system with Philips/ATL L7-4, L12-5, and Vermon L22-14 transducers was used to image human bone marrow-derived stem cells (hBMSCs) cultured in osteogenic media and supersaturated solutions of calcium phosphate. Preliminary results show a 50% increase in Doppler magnitude and a 250% increase in Doppler variance in the presence of cellular mineralization or calcium phosphate crystals compared to images of cells without minerals or in non-crystalline solutions. The L7-4 was found to be more sensitive to twinkling from mineralization than the higher frequency transducers. The increase in Doppler magnitude and variance with cellular mineralization suggests twinkling may be a sensitive early detector of HO. [Work supported by DoD CDMRP PR201164].