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TITLE: Ultrasound-Mediated Nanobiomaterial Delivery for Segmental Bone Fracture Repair

PRINCIPAL INVESTIGATOR: Dan Gazit

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center

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14. ABSTRACT
Severe bone fractures constitute a complex medical condition. Current treatments have significant complications or side effects. We proposed to develop a new technology, which can generate new bone by activating the patient's own stem cells using ultrasound-mediated DNA delivery. We previously showed a proof-of-concept of the technology, named SonoHeal, in a large animal model. In this project, our goals are to determine the optimal delivery device for the injectable DNA and the standard operating procedures for handling and mixing the final product at the clinical site. Furthermore, we aimed to demonstrate the reproducibility and the accuracy of delivering the DNA to the target site. Lastly, we would conduct a toxicology study using the proposed therapy to treat critical-size bone fractures. In the first year of the project, we obtained the necessary approvals to conduct the studies, generated a manual to use the technology in the clinical settings and conducted a study in minipigs to determine the reproducibility and accuracy of DNA delivery.

15. SUBJECT TERMS
Nonunion and segmental fractures, ultrasound, bone regeneration, gene delivery

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1. **INTRODUCTION:**
   Nonunion and segmental bone fractures are caused by trauma and do not heal spontaneously. Treatments include autografts, allografts, the Ilizarov technique, and the use of recombinant Bone Morphogenetic Protein - 2 (BMP-2), all of which involve serious complications or side effects. We proposed a solution, which we named SonoHeal – the use of ultrasound to deliver an osteogenic gene to resident progenitor cells at a fracture site. We had demonstrated statistically significant bone repair, collected initial safety data, and identified the biological mode of action in 59 minipigs. Our overarching objective in this project is to advance SonoHeal to the next go/no go point, namely IND submission to the FDA. We proposed three specific aims**: 1.** Define the delivery method and the standard operating procedure for handling and mixing the final product at the clinical site. **2.** Demonstrate the reproducibility and accuracy of delivery to the target site. **3.** Conduct a toxicology study using the proposed therapy to treat critical-size bone fractures.

2. **KEYWORDS:**
   Nonunion fracture, segmental fractures, gene delivery, fracture healing, resident stem cells, ultrasound.

3. **ACCOMPLISHMENTS:**
   - **What were the major goals of the project?**

Specific Aim 1: Define the microbubble (MB)-DNA delivery device (syringe) and the standard operating procedure (SOP) for handling and mixing the final product at the clinical site.	**Proposed Timeline**	**Actual Timeline**
Major Task 1.1: Development of SOP for preparation of the final product at the clinical site.	Months	Months
Subtask 1.1: Test different sealed sterile vials that will allow easy activation of the MBs and a sterile transfer of the plasmid DNA from its vial to the vial containing activated MBs.	1-3	3
Major Task 1.2: Define the delivery device of the final product.		
Subtask 1.2: Identify the most appropriate syringe to deliver the mixture of MBs and DNA to the fracture, taking into account the volume of the product, distance of target from the skin and visibility of the needle under fluoroscopy imaging.	1-3	3
Milestone(s) Achieved: - Pharmacy manual for preparation of SonoHeal in the clinic -Defined delivery device for SonoHeal injectable component		3
Specific Aim 2: Demonstrate the reproducibility and accuracy of MB-DNA mixture delivery to the target site.		
Major Task 2.1: Submit animal research protocols for Aims 2 and 3.		

Subtask 2.1.1: Update approved animal research protocol with local Institutional Animal Care and Use Committee (IACUC)	0-1	1
Subtask 2.1.3: Submit protocol to Covance Inc. IACUC	3	3
Subtask 2.1.3: Submit protocol to U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP) Animal Care and Use Review Office (ACURO).	1-3	4
Major Task 2.2: Conduct a mini-pig study to demonstrate reproducibility and accuracy (Non-GLP).		
Subtask 2.2.1: Conduct Yucatan minipig segmental fracture surgeries (n=8 total); inject MB-DNA mixture (n=5) or no injection (only surgery)/negative control (n=3).	4	9
Subtask 2.2.2: Implant Duragen matrix in all defects and a metal plate to stabilize the bones (n=8).	4	9
Subtask 2.2.3: On day 14, inject HD-BMP-6 plasmid (1mg) suspended in DEFINITY MBs (10^7) using a syringe appropriate for use with MBs as per manufacturer instructions, following the SOP and using the delivery device determined in Aim 1 on MB-DNA injected minipigs – n=5.	5	9
Subtask 2.2.4: Two days post transfection, all minipigs (n=8) will be sacrificed. Tissue within the defect site will be extracted, digested and subjected to ELISA assay; tissues surrounding the defect site will be analyzed to provide support for accuracy of delivery.	5	11
Milestones Achieved: -IACUC/ACURO approval -Reproducibility and accuracy of SonoHeal injectable material, demonstrated		- Covance IACUC approval (Month 4) - ACURO approvals (Month 6 and 7)
Specific Aim 3: Conduct a GLP toxicology study using the proposed therapy to treat critical-size bone fractures.		
Major Task 3.1: Conduct minipig in vivo surgeries.		
Subtask 3.1.1: Conduct Yucatan minipig segmental fracture surgeries stabilizing tibiae with a custom made 6-hole limited-contact dynamic compression plate and implanting a biodegradable collagen scaffold in the defect site (n=54 total).	6 - 24	19 - Ongoing
Major Task 3.2: Divide minipigs into treatment groups to study and define the SonoHeal safety profile.		
Subtask 3.2.1: Two weeks post-surgery, pigs randomized and assigned to three treatment groups: Group 1) "control" (n=15 male, 15 female); Group 2) "low dose" - minipigs injected with 1mg BMP-6 plasmid suspended in 10^7 MBs [*equivalent to intended maximum clinical dose*] (n=15 male, 15 female); Group 3) "high dose" - minipigs injected with 10mg BMP-6 plasmid suspended in 10^8 MBs [*10-fold greater than*	6-30	19 - Ongoing

intended maximum clinical dose] (n=15 male, 15 female)		
Subtask 3.2.2: Conduct in vivo monitoring and analysis including Cage side clinical observation, weekly physical examination, weight measurements, food consumption monitoring and blood and urine sample collection	6-30	19- Ongoing
Subtask 3.2.3: Conduct analyses on blood and urine samples collected during in vivo monitoring.	6-30	
Major Task 3.3: Conduct postmortem tests		
Subtask 3.3.1: Conduct histopathology analyses on animals sacrificed at designated time points (3 days, 3 months, 9 months) by collecting tissue samples of injection site, brain, bone marrow (femur), heart, kidney, liver, lung, lymph nodes, ovary, testis, and spleen. For each experimental group: n=3M/3F (3 days), n=3M/3F (3 months), n=3M/3F (9 months).	6-30	30-Ongoing
Subtask 3.3.2: Conduct biodistribution analyses on animals sacrificed at designated time points (3 days, 3 months, 9 months) by collecting tissue samples of injection site, brain, bone marrow (femur), heart, kidney, liver, lung, lymph nodes, ovary, testis, and spleen as well as blood for ELISA assay for BMP-6 detection; quantitative RT-PCR for the detection of BMP-6 expression; and X-ray imaging to rule out ectopic bone formation. For each experimental group: n=3M/3F (3 days), n=3M/3F (3 months), n=3M/3F (9 months).	6-30	30-Ongoing
Subtask 3.3.3: Evaluate fracture union on tibia bones using a microCT scanner at designated time points (3 days, 3 months, 9 months). For the experimental groups: n=6M/6F (3 months), n=6M/6F (9 months).	6-30	30-Ongoing
Subtask 3.3.4: Conduct biomechanical testing at designated time points (3 months, 9 months). For each experimental group: n=6M/6F (3 months), n=6M/6F (9 months).	12-36	
Milestone(s) Achieved: -Toxicology, tumorigenicity and biodistribution results of SonoHeal -Results of segmental defect fracture union at various doses of BMP-6 plasmid -Biomechanical evaluation results of segmental fracture repair -Documentation of project results for IND filing		

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

1. Major activities:

- **Reproducibility and accuracy study in mini pigs:** A study on 8 minipigs was conducted at Covance Laboratories Inc. New mass spectrometry assay was developed for the detection of human BMP-6 (hBMP-6).

- **GLP toxicology study in mini pigs:** A study was initiated, and 37 animals were operated and treated so far. 35 animals have been euthanized and samples collected for biodistribution, histopathology and microCT analyses.

2. Specific objectives:
   i. **Conduct a mini-pig study to demonstrate reproducibility and accuracy (non-GLP)**
   ii. **Conduct a GLP toxicology study using the proposed therapy to treat critical-size bone fractures.**

3. Significant results:
   i. In the previous report period, we established a mass spectrometry assay to detect a surrogate peptide that is unique to human BMP-6, which should allow us to analyze hBMP-6 biodistribution in pig tissues. Various measures, including the increase of the volume of the samples to 1ml, were used in order to increase the sensitivity of the assay. Final results indicated that the lower limit of quantification (LLOQ) was determined to be 0.2 ng/ml. The method will be used to determine the biodistribution of human BMP-6 protein in the tissues of the pigs in the toxicology study.

   ii. A GLP toxicology study was initiated at Covance Laboratory CRO. Thirty-six mini-pigs (Groups 2, 3, 5, 6, 8 and 9 – **Table 1**) were operated and a 1-cm bone defect was created in their tibia bones. A collagen scaffold (Duragen) was placed in all defects. The animals were randomized to receive low dose injection of hBMP-6 plasmid and microbubbles (1mg and 10^7), high dose (10mg and 10^8) and a negative control that was not injected. Each group consisted of six animals (3F;3M) and was monitored for 3 and 9 months. Blood and urine were collected from all animals, pre-treatment and at the end of the study.

Table 1: GLP Toxicology Experimental Groups

Group	Treatment	Scaffold implantation	Dose per injection	Number of animals (M/F)	Scheduled Termination
1	Control	Yes	0	3/3	3 Days
2	Control	Yes	0	3/3	3 Months
3	Control	Yes	0	3/3	9 Months
4	Low dose	Yes	1 mg DNA and 10^7 MBs	3/3	3 Days
5	Low dose	Yes	1 mg DNA and 10^7 MBs	3/3	3 Months
6	Low dose	Yes	1 mg DNA and 10^7 MBs	3/3	9 Months
7	High dose	Yes	10 mg DNA and 10^8 MBs	3/3	3 Days
8	High dose	Yes	10 mg DNA and 10^8 MBs	3/3	3 Months
9	High dose	Yes	10 mg DNA and 10^8 MBs	3/3	9 Months

Following euthanasia, tissue samples were collected from all animals by the study's pathologist. The samples will be used for histopathology and biodistribution analysis.

Operated tibia bones were also collected and subjected to microCT imaging and analyses.

Results (midterm, non-final report):

1. There was one early death after treatment in the 9-month group, which was attributed left heart failure due to a cardiac infarct of unknown origin but was presumed to be a spontaneous event not related to treatment. Additional pig was added to the study to replace this animal.
   From a clinical perspective, most animals survived to the designated endpoint without significant adverse clinical signs. Most animals experienced mild-moderate lameness, which lasted 1-2 months in most cases. There were no obvious treatment related effects as they related to animal health or signs of lameness. There were two animals, both in the 9-month group which had broken locking compression plates which did not cause serious lameness. Both were carried to term and did clinically well. One pig had longer than usual lameness which was likely exacerbated by a treatment site infection, which required flushing and antibiotic therapy. This animal survived to study term. In summary, no significant adverse treatment related events were observed.
   The clinical pathology (hematology and serum chemistry) samples were analyzed at baseline, pre-termination as well as one data point between baseline and termination for all animals in the study. The hematology and serum chemistry results were within normal reference ranges indicating the pigs were in good health during the duration of the study. A few isolated outside the normal ranges were seen, however, these few observations were deemed clinical inconsequential. Urinalysis was conducted at baseline and termination for all animals and the results demonstrated largely normal for all study animals to date.
2. A mistake in animal distribution occurred in the 3-months groups. Female and male animals were not evenly distributed between the groups. Additional four animals will be added to the study to compensate for this mistake.
3. MicroCT imaging and analysis of operated tibia bones showed that also control animals healed in the 3 and 9-months group. This could be attributed to spontaneous bone formation in adolescent minipigs. Hence, it was difficult to determine the efficacy of the treatment, as shown in **Figures 1 & 2**. [BV= bone volume; BMD = bone mineral density; BMC = Bone mineral content; gm=gram].
   Based on these results, we will determine the definitive efficacy of the treatment in the sheep study conducted with NCATS/NIH (see below). The results of the GLP study will be focused on the toxicology and safety of the treatment.

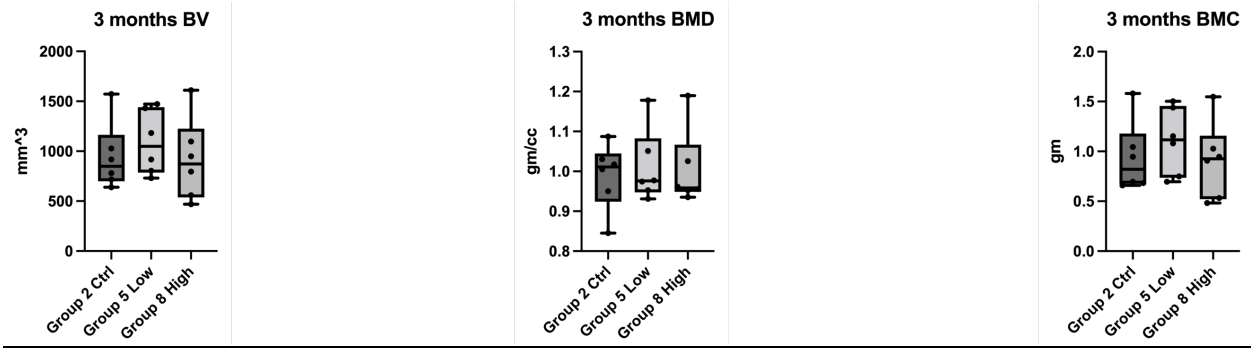

Figure 1: 3-month groups - microCT analyses

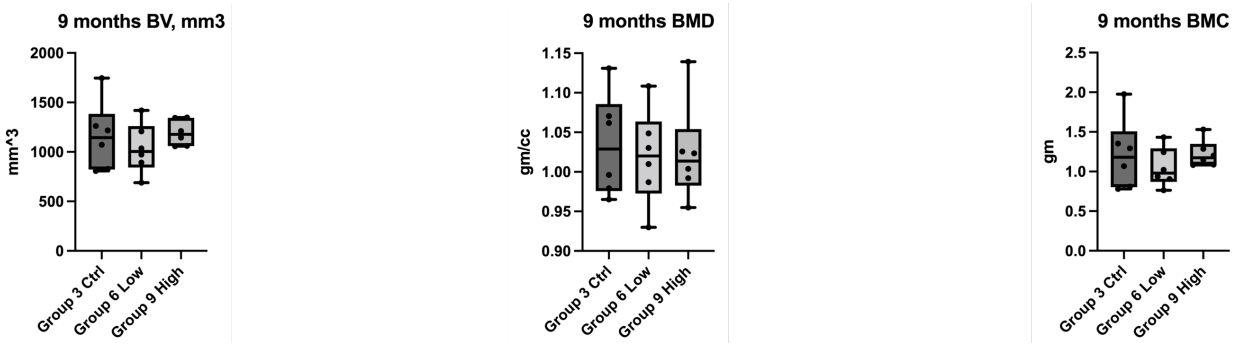

Figure 2: 9-months group - microCT analyses

- **What opportunities for training and professional development has the project provided?**
  Nothing to report.

- **How were the results disseminated to communities of interest?**
  Nothing to report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  In the next report period (a no-cost extension), we intend to complete surgeries and treatment of four animals in the 3-months groups (#2, 5, 6) and 18 animals in the 3-day groups (#1, 4, 7). Following euthanasia, we plan to complete the histopathology and biodistribution analyses of all animals,

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
   - **What was the impact on the development of the principal discipline(s) of the project?**
     Nothing to report.

   - **What was the impact on other disciplines?**
     Nothing to report.

   - **What was the impact on technology transfer?**
     In the previous report we disclosed the collaborative research agreement we signed with NIH National Center for Advancing Translational Sciences (NCATS) within the Bridging Interventional Development Gaps (BrIDGs) program. As part of this collaboration a sheep study of 41 animals was initiated and is ongoing. The goal of the study is to determine the efficacy of the treatment in large bone defects (3 cm compared to 1 cm in the pig model). GMP production of the human BMP-6 plasmid was contracted and is about to begin.

   - **What was the impact on society beyond science and technology?**
     Nothing to report.

5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
    - **Changes in approach and reasons for change**
      Additional animals were added to the GLP study, as explained above.

    - **Actual or anticipated problems or delays and actions or plans to resolve them**
      We requested a 1-year no-cost extension to complete the GLP study and analyze the results.

    - **Changes that had a significant impact on expenditures**
      Nothing to report.

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

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

    Publications, conference papers, and presentations
    Nothing to report.

    Website(s) or other Internet site(s)
    Nothing to report.

    Technologies or techniques
    Nothing to report.

    Inventions, patent applications, and/or licenses
    Cedars-Sinai Medical Center is negotiating a license agreement with SonoStem Technologies Inc., which aims to further develop the technology and commercialize it.

    Other Products
    Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
    - **What individuals have worked on the project?**

Name:	Dan Gazit	Gadi Pelled	Zulma Gazit	Pablo Avalos
Project Role:	PI	Co-investigator	Co-Investigator	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	https://www2.scopus.com/authid/detail.uri?authorId=7005550705	https://www.scopus.com/authid/detail.uri?authorId=6602319400	https://www2.scopus.com/authid/detail.uri?auth	https://www2.scopus.com/authid/detail.uri?aut

			orId=6602611025	horId=12545883000
Nearest person month worked:	4	4	4	1
Contribution to Project:	PI, oversees all aspects of the project	Study coordinator	Oversees all lab work	Animal surgeon
Funding Support:	This project	This project	This project	Internal funding from Cedars-Sinai

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  Dr. Dan Gazit was awarded a 4-year NIH R01 (2R01AR066517 - 05A1). The project aims to develop new MRI-based methods for the diagnosis of low back pain. No overlap exits with this project. He will dedicate 15% effort (1.8 calendar) to this project. Dr. Zulma Gazit will be a co-investigator in this project and dedicate 15% effort (1.8 calendar) as well. No other changes in the active support of the other personnel.

- **What other organizations were involved as partners?**
  - **Organization Name:** LabCorp Early Development Laboratories Inc. (Previously - Covance Laboratories Inc.), a contract research organization for pre-clinical studies.
  - **Location of Organization:** San Carlos, CA.
  - **Partner's contribution to the projec**t
    - **Other:** Animal studies were performed at Covance Laboratories under a service agreement with Cedars-Sinai.
  - **Organization Name:** AIT Bioscience Inc., a contract research organization for pre-clinical and clinical studies with specialization in analytical assays.
  - **Location of Organization:** Indianapolis, IN
  - **Partner's contribution to the projec**t
    - **Other:** AIT performed ELISA and mass spectrometry assays under a service agreement with Covance Laboratories and Cedars-Sinai.
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8. **Special Reporting Requirements**

   None.

9. **Appendices**

   None.