AWARD NUMBER: W81XWH-18-1-0593

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

PRINCIPAL INVESTIGATOR: Dan Gazit

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center

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6. AUTHOR(S)

Dan Gazit

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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13. SUPPLEMENTARY NOTES

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 40 animals were operated and treated so far. 40 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. 40 mini-pigs (Groups 2, 3, 5, 6, 8 and 9, plus 4 replacement pigs – **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 were added and treated 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.

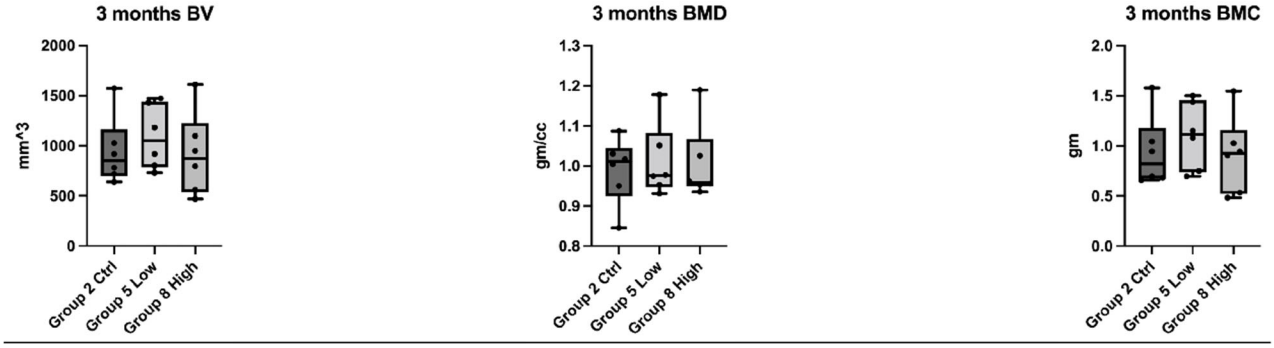

Figure 1: 3-month groups - microCT analyses

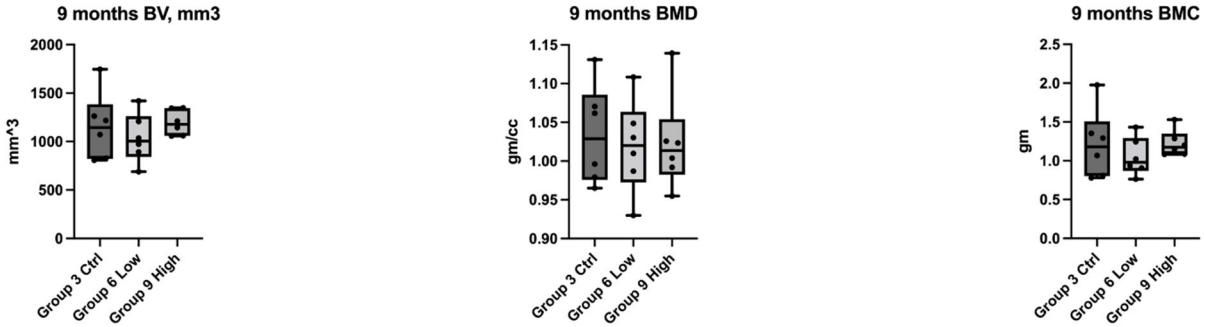

Figure 2: 9-months group - microCT analyses

- **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?**
  Nothing to report

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, we began a sheep study of 41 animals to determine the efficacy of the treatment in large bone defects (3 cm compared to 1 cm in the pig model). The surgeries and the in-life portion of the study was completed, and the analysis is underway. 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**
      Early termination of award effective 06/16/2022 due to PI, Dan Gazit and Co-I's, Zulma Gazit and Gadi Pelled leaving Cedars-Sinai Medical Center.

    - **Actual or anticipated problems or delays and actions or plans to resolve them**

    - **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
Nearest Person Month Worked:	6	6	4	1
Contribution to Project:	PI	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?**
  Nothing to report

- **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 project**
    - **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 project**
    - **Other:** AIT performed ELISA and mass spectrometry assays under a service agreement with Covance Laboratories and Cedars-Sinai.

8. Special Reporting Requirements

None.

9. Appendices

None.

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PRINCIPAL INVESTIGATOR: Dan Gazit

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6. AUTHOR(S)
Dan Gazit and Gadi Pelled

E-Mail: dan.gazit@csmc.edu

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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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   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.		
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Milestone(s) Achieved: - Pharmacy manual for preparation of SonoHeal in the clinic -Defined delivery device for SonoHeal injectable component		3
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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
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- **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.

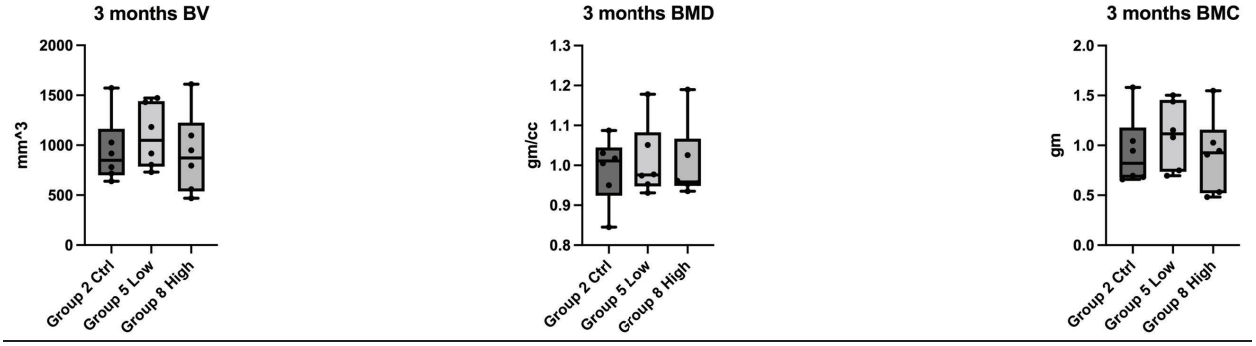

Figure 1: 3-month groups - microCT analyses

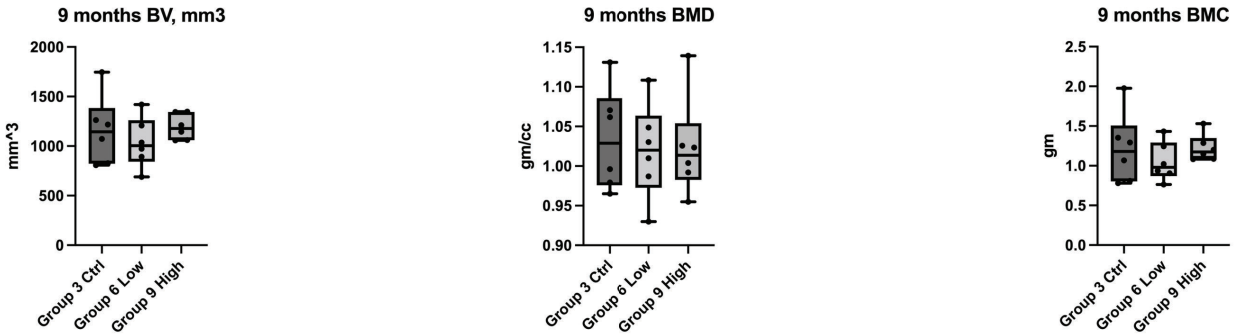

Figure 2: 9-months group - microCT analyses

- **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.
    -

8. **Special Reporting Requirements**

   None.

9. **Appendices**

   None.

AWARD NUMBER: W81XWH-18-1-0593

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

PRINCIPAL INVESTIGATOR: Dan Gazit

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center

REPORT DATE: 10/14/2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dan Gazit and Gadi Pelled

E-Mail: dan.gazit@csmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Los Angeles, CA 90048-1804

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13. SUPPLEMENTARY NOTES

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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=90 total).	6 - 24	19
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

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	19-28
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	
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	
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	
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 18 animals were operated and treated.

2. Specific objectives:

i. **Conduct a mini-pig study to demonstrate reproducibility and accuracy (Non-GLP) -** study was conducted. Results of biochemical assays (ELISA) are being processed.

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 conducted an accuracy and reproducibility study of SonoHeal's gene transfer to bone fracture sites. Eight minipigs (4 females and 4 males; 35-50 kg; age-7 months) were operated according to the approved protocols and a 1-cm segmental defect was created in their one of their tibia bones. A Duragen scaffold (Integra LifeSciences) was placed in the defect to attract resident stem cells. The tibiae were stabilized using a locking plate and six screws. Two weeks post operation, the animals were anesthetized and a mixture of BMP-6 plasmid DNA and microbubbles (Definity) was injected to the defect site under fluoroscopic guidance. Immediately after injection ultrasound pulse was applied over the defect site to oscillate the microbubbles, for two minutes or until no bubbles were visible.

Two- and three-days post DNA delivery, the animals were euthanized, and tissues were collected from the defect site and from soft tissues surrounding the defect (medial, lateral and posterior). In addition, soft tissue was collected from the contra lateral limb and from a minipig that was not included in the study (obtained from a non-survival study conducted at Cedars Sinai at that time). All tissues were frozen in -80°c for at least 24 hours and shipped on dry ice to AIT Bioscience Inc.

At AIT the tissues were thawed, homogenized and suspended in proteinase inhibitor buffer according to the BMP-6 ELISA manufacturer instructions (R&D Systems). ELISA

Minipig Bone Marrow, Soft Tissue, Contralateral Leg Tissue, and Serum PK for BMP-6

Sample Lot ID	Description	Sample	Generated Value (pg/mL)	Result (pg/mL)	Dilution Factor	Dilution Corrected Result (pg/mL)	Spiked Sample ID	Spike Result (pg/mL)	Run	Nominal Spike (pg/mL)	Spike Recovery %	Sample BCA Result (mg/mL)	Reportable Result (pg/mg)	Day post sonoporation
2457-7517-F-BM	Bone Marrow within defect	S1	36.4	BLQ<(50.0)	20	<1000	S1 SPK	86.1	0001-029	78	64	4.8	<209	
2457-7517-F-MS	Medial soft tissue	S2	15.2	BLQ<(50.0)	20	<1000	S2 SPK	74.7	0001-031	78	76	3.5	<286	
2457-7517-F-PS	Posterior soft tissue	S3	12.8	BLQ<(50.0)	20	<1000	S3 SPK	74.7	0001-033	78	79	1.7	<572	3
2457-7517-F-CL	Contralateral Leg	S4	356	356	20	7120	S4 SPK	386	0001-035	78	38	7.7	924.1	
2457-7517-F-LS	Lateral soft tissue	S5	0	BLQ<(50.0)	20	<1000	S5 SPK	51.5	0001-037	78	66	2.2	<465	
2457-7517-F-S	Serum	S6	0	BLQ<(50.0)	20	<1000	S6 SPK	BLQ<(50.0)	0001-039	78	N/A	56.1	<18	
2457-7517-F-S	Serum	S6	0	BLQ<(50.0)	20	<1000	S6 SPK	BLQ<(50.0)	0003-001	78	N/A	56.1	<18	
2457-7517-F-S	Serum	S6 40X	0	BLQ<(50.0)	40	<2000	S6 40X SPK	BLQ<(50.0)	0003-069	78	N/A	56.1	<36	
2457-7517-F-S	Serum	S6 60X	0	BLQ<(50.0)	60	<3000	S6 60X SPK	BLQ<(50.0)	0003-073	78	N/A	56.1	<53	
2457-7517-F-S	Serum	S6 80X	7.22	BLQ<(50.0)	80	<4000	S6 80X SPK	16.2	0003-077	78	12	56.1	<71	
2457-7664-A-BM	Bone Marrow within defect	S7	22	BLQ<(50.0)	20	<1000	S7 SPK	77.8	0001-041	78	72	5.1	<196	
2457-7664-A-MS	Medial soft tissue	S8	0	BLQ<(50.0)	20	<1000	S8 SPK	46.2	0001-043	78	59	4.5	<221	
2457-7664-A-PS	Posterior soft tissue	S9	0	BLQ<(50.0)	20	<1000	S9 SPK	65.3	0001-045	78	84	1.2	<834	2
2457-7664-A-CL	Contralateral Leg	S10	1070	1070	20	21400	S10 SPK	1180	0001-047	78	141	1.0	20736.4	
2457-7664-A-LS	Lateral soft tissue	S11	0	BLQ<(50.0)	20	<1000	S11 SPK	72.6	0001-049	78	93	1.2	<822	
2457-7228-B-BM	Bone Marrow within defect	S12	4.36	BLQ<(50.0)	20	<1000	S12 SPK	74.7	0001-051	78	90	3.8	<261	
2457-7228-B-MS	Medial soft tissue	S13	0	BLQ<(50.0)	20	<1000	S13 SPK	71.6	0001-053	78	92	1.2	<825	
2457-7228-B-PS	Posterior soft tissue	S14	0	BLQ<(50.0)	20	<1000	S14 SPK	85.1	0001-055	78	109	2.1	<481	2
2457-7228-B-CL	Contralateral Leg	S15	116	116	20	2320	S15 SPK	234	0001-057	78	151	1.3	1836.9	
2457-7228-B-LS	Lateral soft tissue	S16	28.7	BLQ<(50.0)	20	<1000	S16 SPK	118	0001-059	78	114	0.8	<1244	
2457-7270-E-BM	Bone Marrow within defect	S17	231	231	20	4620	S17 SPK	306	0004-001	78	96	6.8	679.1	
2457-7270-E-MS	Medial soft tissue	S18	6.33	BLQ<(50.0)	20	<1000	S18 SPK	89.9	0004-003	78	107	1.1	<943	
2457-7270-E-PS	Posterior soft tissue	S19	0	BLQ<(50.0)	20	<1000	S19 SPK	68.2	0004-005	78	87	1.8	<569	3
2457-7270-E-CL	Contralateral Leg	S20	444	444	20	8880	S20 SPK	555	0004-007	78	142	2.0	4544.5	
2457-7270-E-LS	Lateral soft tissue	S21	0	BLQ<(50.0)	20	<1000	S21 SPK	93.3	0004-009	78	120	5.4	<186	
2457-7720-G-BM	Bone Marrow within defect	S22	2.29	BLQ<(50.0)	20	<1000	S22 SPK	72.3	0004-011	78	90	1.8	<555	
2457-7720-G-MS	Medial soft tissue	S23	25.1	BLQ<(50.0)	20	<1000	S23 SPK	100	0004-013	78	96	1.0	<1020	
2457-7720-G-PS	Posterior soft tissue	S24	25.1	BLQ<(50.0)	20	<1000	S24 SPK	95.8	0004-015	78	91	0.9	<1140	3
2457-7720-G-CL	Contralateral Leg	S25	208	208	20	4160	S25 SPK	320	0004-017	78	144	3.0	1383.4	
2457-7720-G-LS	Lateral soft tissue	S26	557	557	20	11140	S26 SPK	644	0004-019	78	112	2.2	5110.1	
2457-8175-C-BM	Bone Marrow within defect	S27	4.71	BLQ<(50.0)	20	<1000	S27 SPK	74.8	0004-021	78	90	6.2	<161	
2457-8175-C-MS	Medial soft tissue	S28	2.29	BLQ<(50.0)	20	<1000	S28 SPK	69.8	0004-023	78	87	1.1	<923	
2457-8175-C-PS	Posterior soft tissue	S29	31.7	BLQ<(50.0)	20	<1000	S29 SPK	103	0004-025	78	91	0.8	<1247	2
2457-8175-C-CL	Contralateral Leg	S30	64	64	20	1280	S30 SPK	181	0004-027	78	150	1.4	924.9	
2457-8175-C-LS	Lateral soft tissue	S31	30.8	BLQ<(50.0)	20	<1000	S31 SPK	127	0004-029	78	123	1.8	<546	
2457-7252-H-BM	Bone Marrow within defect	S32	6.33	BLQ<(50.0)	20	<1000	S32 SPK	69	0004-031	78	80	3.9	<254	
2457-7252-H-MS	Medial soft tissue	S33	0	BLQ<(50.0)	20	<1000	S33 SPK	52.8	0003-003	78	68	2.2	<451	
2457-7252-H-PS	Posterior soft tissue	S34	0	BLQ<(50.0)	20	<1000	S34 SPK	82.7	0003-005	78	106	5.5	<183	3
2457-7252-H-CL	Contralateral Leg	S35	185	185	20	3700	S35 SPK	254	0003-007	78	88	5.9	623.5	
2457-7252-H-LS	Lateral soft tissue	S36	99.7	99.7	20	1994	S36 SPK	202	0003-009	78	131	6.3	315.8	
2457-7266-D-BM	Bone Marrow within defect	S37	10.9	BLQ<(50.0)	20	<1000	S37 SPK	64.8	0003-011	78	69	7.7	<131	
2457-7266-D-MS	Medial soft tissue	S38	0	BLQ<(50.0)	20	<1000	S38 SPK	61.4	0003-013	78	79	0.6	<1621	
2457-7266-D-PS	Posterior soft tissue	S39	0	BLQ<(50.0)	20	<1000	S39 SPK	58	0003-015	78	74	0.7	<1502	2
2457-7266-D-CL	Contralateral Leg	S40	281	281	20	5620	S40 SPK	348	0003-017	78	86	1.1	5237.7	
2457-7266-D-LS	Lateral soft tissue	S41	0	BLQ<(50.0)	20	<1000	S41 SPK	66.5	0003-019	78	85	0.5	<1866	

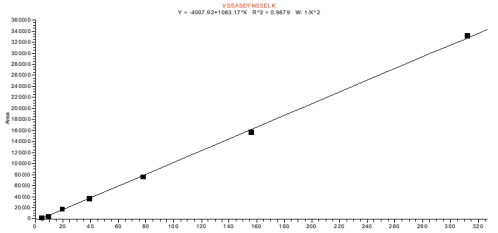
Figure 1: hBMP-6 ELISA Results

assay was carried out using various buffers and conditions to determine the optimal protocol for BMP-6 quantification. Unfortunately, the results did not make biological sense as HBMP-6 was detected mainly in the contralateral legs and even in tissues collected from untreated animals (**Fig 1**). Following discussions with bioanalysis experts, it was concluded that the ELISA assay might cross react with porcine BMP-6. Hence, it was recommended to try to detect the protein using mass spectrometry. Analysis of predicted tryptic peptides revealed a peptide that was unique to the human BMP-6 protein: VSSASDYNSSELK (in comparison to the porcine BMP-6).
Human BMP-6 was reduced using triethyl phosphine and digested using trypsin overnight. Samples were ran on a High Resolution Mass Spectrometer (HRMR) and serial dilutions in buffer resulted in 5ng/ml detection sensitivity (**Fig. 2**)

Protocol

1. Perform serial dilution of human BMP-6 protein
2. Reduce each sample with triethyl phosphine (TEP)
3. Digest overnight with trypsin
4. Inject to High Resolution Mass Spectrometer (HRMS)
5. Detect VSSASDYNSSELK peptide by full scan MS and determine calibration curve

Standard Curve (5-300 ng/ml) shown



Standard Curve Statistics

Sample Name	Area	Specified Amount ng/ml	Calculated Amount ng/ml	%Diff
4.9	1667	4.900	5.337	9%
9.8	4427	9.800	7.934	-19%
19.5	17755	19.500	20.470	5%
39	36288	39.000	37.901	-3%
78	75536	78.000	74.818	-4%
156	156769	156.000	151.224	-3%
312	332143	312.000	316.177	1%
1250	1449375	1250.000	1367.028	9%
2500	2411458	2500.000	2271.947	-9%
5000	6030756	5000.000	5676.198	14%

Chromatograms 5-40 ng/ml

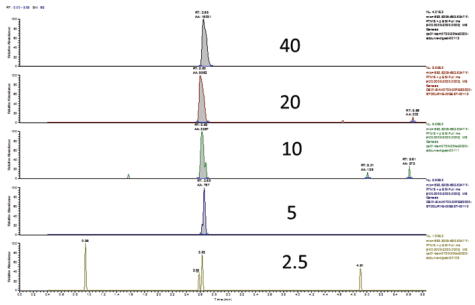
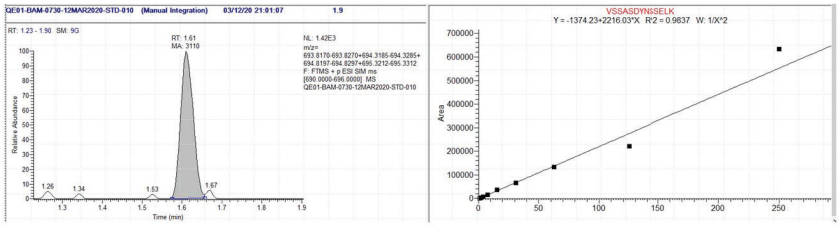


Figure 2: HRMR results of hBMP-6 digestion

Next, the detection of hBMP-6 using the surrogate peptide was attempted in matrix (homogenized pig muscle tissue). It was found that immunoprecipitation of the protein was required prior to digestion in order to detect the peptide (**Fig. 3**).
An attempt was made to increase the sensitivity of the assay by using a triple quadrupole mass spectrometer

Neat (Buffer)
Immunoprecipitation prior to Trypsin Digestion



Homogenized Matrix
Immunoprecipitation prior to Trypsin Digestion

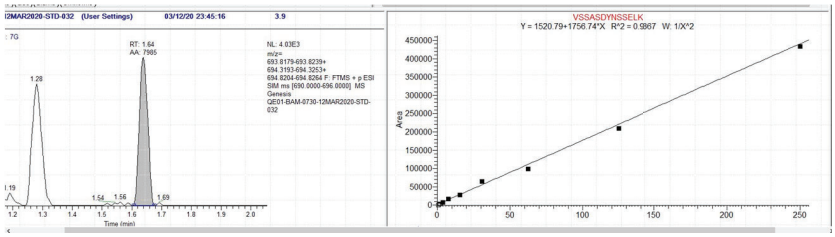


Figure 3: Detection of Surrogate Peptide in Matrix

(Thermo Scientific TSQ Quantiva), however the results did not differ from those obtained with HMRM. In addition, we increased the volume of the sample by 10X (to 500ul) and compared the use of two antibodies for hBMP-6 immunoprecipitation (manufactured by Boster and R&D). The R&D polyclonal antibody was found to have slightly better results at the low end of the curve (**Fig. 4**).

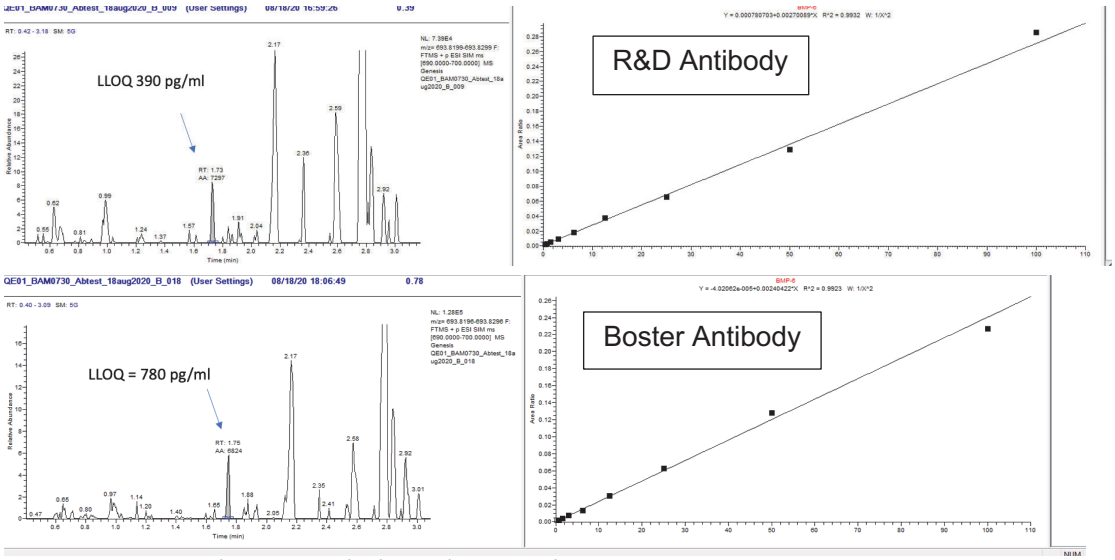


Figure 4: Compassion between antibodies and 10X samples

In summary, 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. We are currently increasing the volume of the samples to 1ml, which should enable us to reach a sensitivity of 100pg/ml. The analysis will be done on samples obtained from the 8-minipig study of reproducibility and accuracy.

ii. A GLP toxicology study was initiated at Covance Laboratory CRO. Eighteen mini-pigs (Groups 3, 6 and 9 – **Table 1**) were operated and a 1-cm bone defect was created in their tibia bones (**Fig. 5**). 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 will be monitored for nine months (till Month 28 of the project).

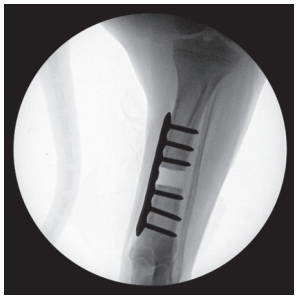


Figure 5: Mini-pig tibia osteotomy (X-ray)

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

- **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, we intend to complete final the mass spectrometry results of Aim 1and complete Specific Aim 3. Specifically, we will operate and treat animals from Groups 1,2,4,5,7&8 (**Table 1**). Following euthanasia, we will analyze all the results obtained during the GLP study.

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?**
     We established a mass spectrometry assay to detect human BMP-6 in porcine tissues that would replace the ELISA assay we originally aimed to use.

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

   - **What was the impact on technology transfer?**
     We have reached a collaborative research agreement with NIH National Center for Advancing Translational Sciences (NCATS) within the Bridging Interventional Development Gaps (BrIDGs) program. Our collaborators at NCATS will fund and perform additional pre-IND activities with SonoHeal that will include i. An efficacy study in sheep; ii Product stability tests; iii. GMP production of biological material for clinical trials, and iv. Assist with IND submission to the FDA.

   - **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**
      As reported above, the ELISA assay we had aimed to use in order to assess biodistribution, did not accurately detect human BMP-6 in porcine tissues. Hence, we had to develop a mass spectrometry assay that would detect a unique peptide in the human protein.

    - **Actual or anticipated problems or delays and actions or plans to resolve them**
      The need to develop a new bioanalytical assay did result in some delay, but we expect to complete all aims on time.

    - **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
    Nothing to report.

    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=700555070 5	https://orcid.org/0000-0003-3857-5531	https://www2.scopus.com/authid/detail.uri?authorId=660261102 5	https://www2.scopus.com/authid/detail.uri?authorId=1254588 3000
Nearest person month worked:	8	8	7	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?**
  Nothing to report.
- **What other organizations were involved as partners?**
  - **Organization Name:** 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.
    -

8. Special Reporting Requirements

None.

9. Appendices

None.

AWARD NUMBER: W81XWH-18-1-0593

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

PRINCIPAL INVESTIGATOR: Dan Gazit

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center

REPORT DATE: 10/13/2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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5b. GRANT NUMBER
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5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8700 Beverly Blvd.
Los Angeles, CA 90048-1804

8. PERFORMING ORGANIZATION REPORT NUMBER

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12. DISTRIBUTION / AVAILABILITY STATEMENT
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13. SUPPLEMENTARY NOTES

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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	
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=90 total).	6 - 24	
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	

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	
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	
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	
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	
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:

- **Change of SOW:** based on the advice of our regulatory consultant, we aimed to conduct the major part of the animal studies included in the project, under GLP conditions. We received the approval of our Science Officer and a modification to the SOW was

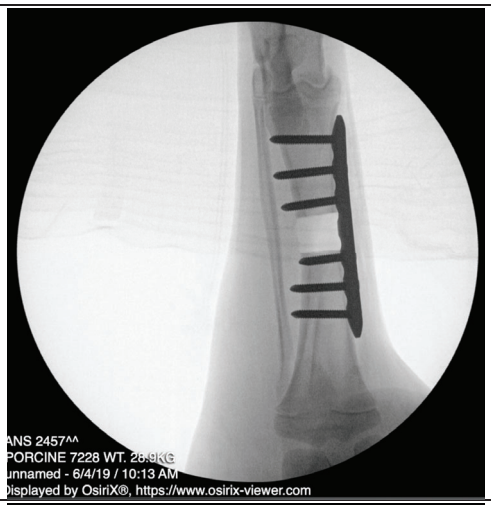
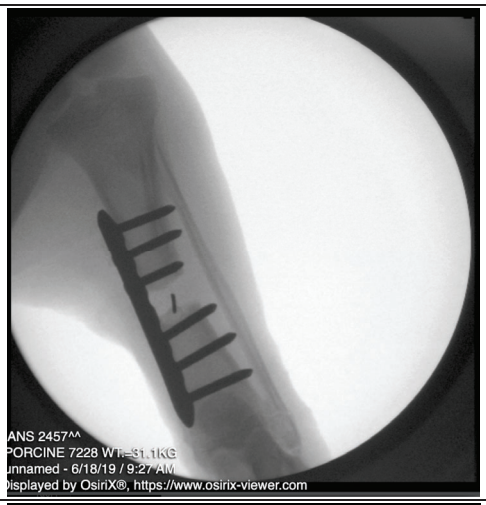
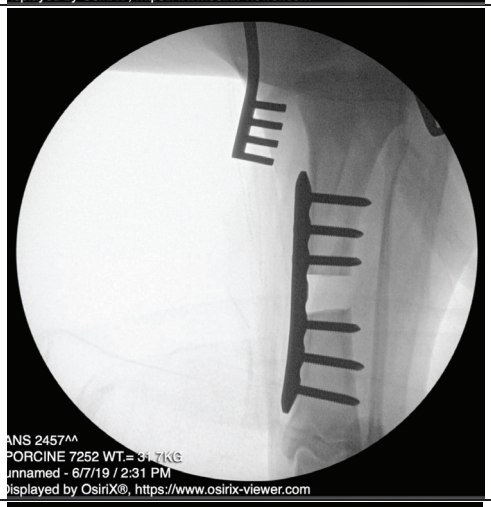
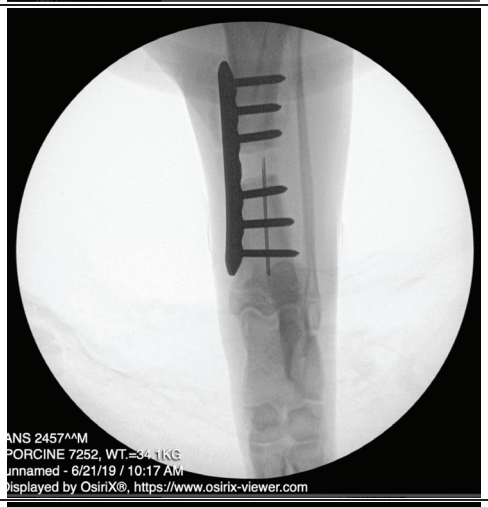
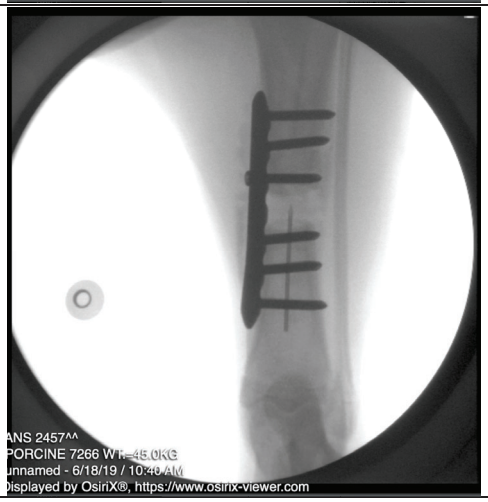
approved on Month 2. The GLP studies will be performed at a qualified Contract Research Organization (CRO), Covance Laboratories Inc., in San Carlos, CA.

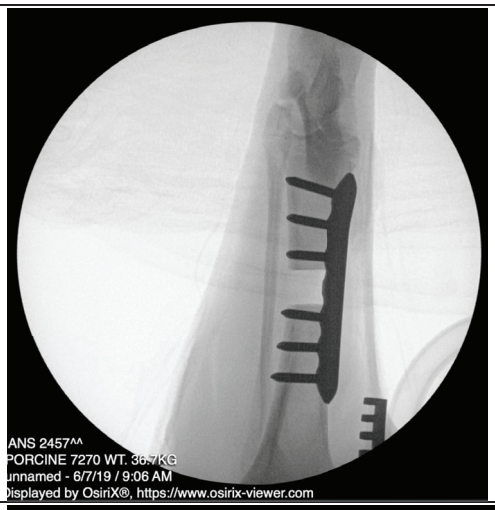
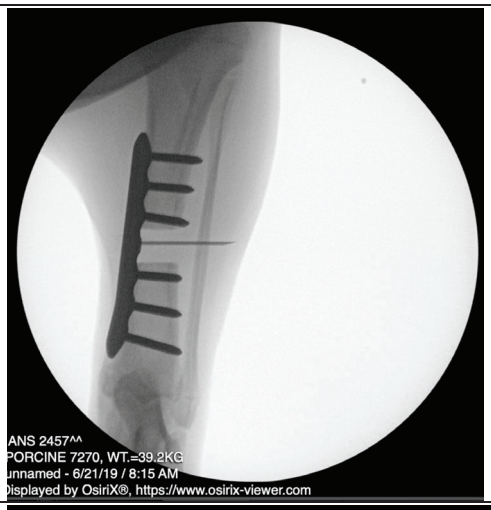
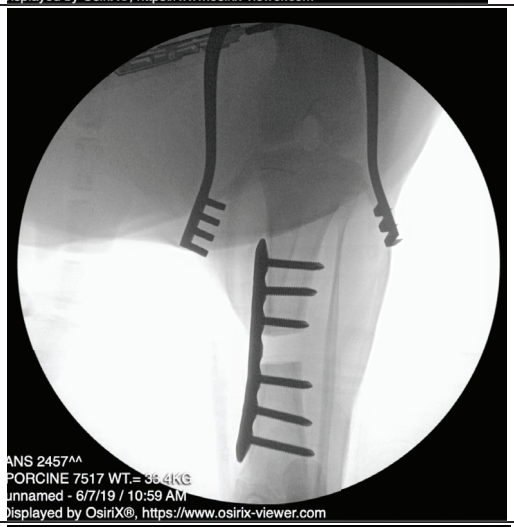
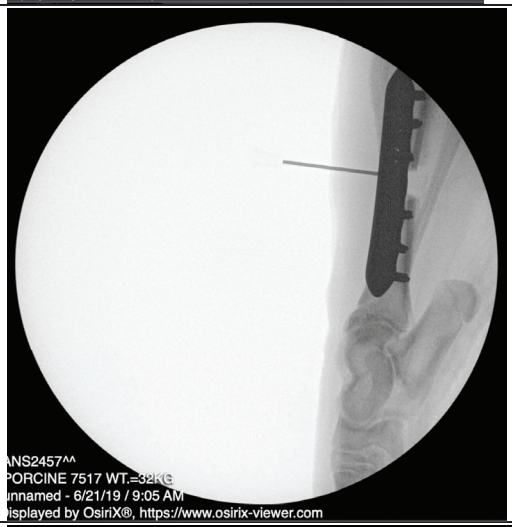
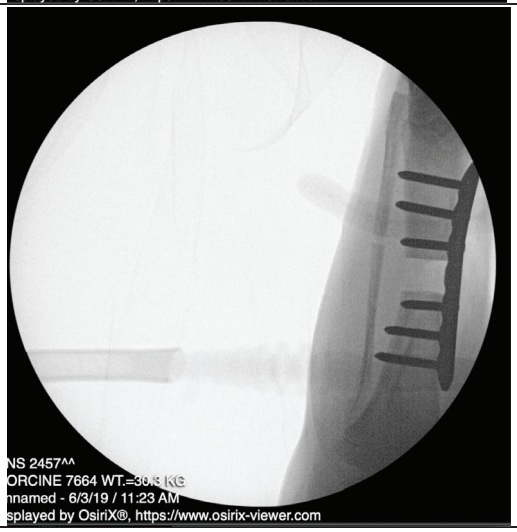
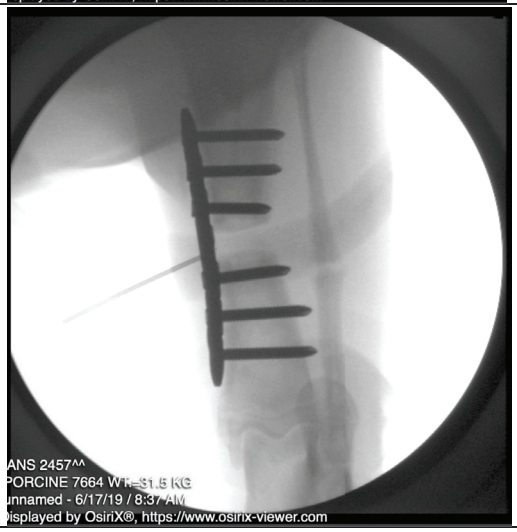
- **Submission of study protocols**: Animal study protocols were submitted to the IACUC at Cedars-Sinai Medical Center and at Covance Laboratories Inc. Following the approval of the protocols a submission was made to ACURO.
- **SOP for preparation of final product / Pharmacy manual:** we generated two SOPs – one for the animal studies and another for future clinical use. The animal SOP was used in the initial study to demonstrate reproducibility and accuracy of gene delivery.
- **Reproducibility and accuracy study in mini pigs:** A study on 8 minipigs was conducted at Covance Laboratories Inc.

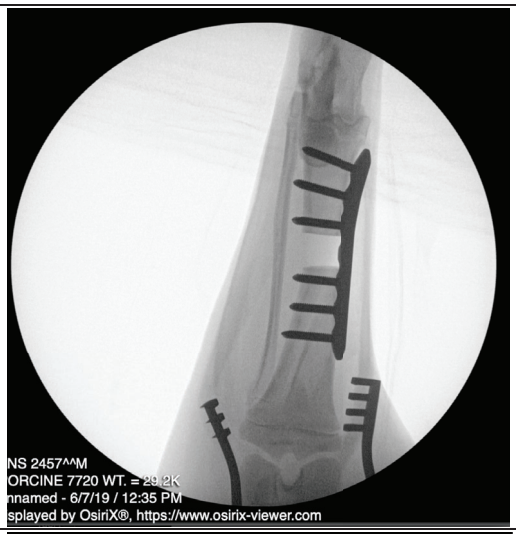
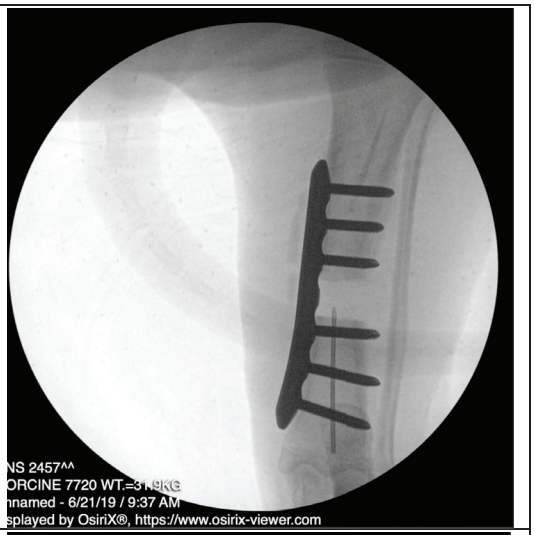
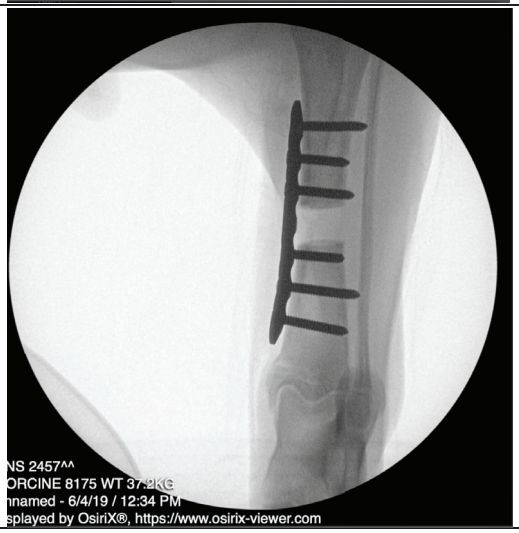
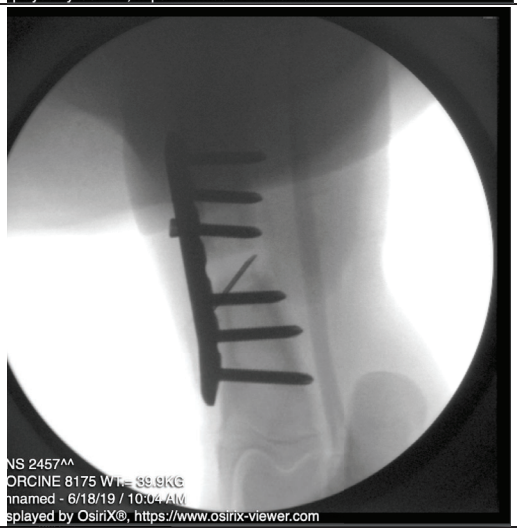
2. Specific objectives:
   i. **Protocol approvals:** all protocols were approved by Month 7.
   ii. **Development of SOP for preparation of the final product at the clinical site:** SOP was generated.
   iii. **Define the delivery device of the final product –** Device was defined and included in the SOP.
   iv. **Conduct a mini-pig study to demonstrate reproducibility and accuracy (Non-GLP) -** study was conducted. Results of biochemical assays (ELISA) are being processed.

3. Significant results:
   In order to determine the accuracy and reproducibility of SonoHeal's gene transfer to bone fracture sites, we conducted a study in minipigs. Eight minipigs (4 females and 4 males; 35-50 kg; age-7 months) were operated according to the approved protocols and a 1-cm segmental defect was created in their one of their tibia bones. A Duragen scaffold (Integra LifeSciences) was placed in the defect to attract resident stem cells. The tibiae were stabilized using a locking plate and six screws (**Table 1**). Two weeks post operation, the animals were anesthetized and a mixture of BMP-6 plasmid DNA (high documented) and microbubbles (Definity) was injected to the defect site under fluoroscopic guidance (**Table 1; note needle delivering BMP-6 and microbubbles seen in the defects)**. Immediately after injection ultrasound pulse was applied over the defect site to oscillate the microbubbles, for two minutes or until no bubbles were visible. Two- and three-days post DNA delivery, the animals were euthanized, and tissues were collected from the defect site and from sites surrounding the defect (medial, lateral and posterior). In addition, soft tissue was collected from the contra lateral limb and from a minipig that was not included in the study (obtained from a non-survival study conducted at Cedars Sinai at that time). All tissues were frozen in -80°c for at least 24 hours and shipped on dry ice to AIT Bioscience Inc.
   At AIT the tissues were thawed, homogenized and suspended in proteinase inhibitor buffer according to the BMP-6 ELISA manufacturer instructions (R&D Systems). ELISA was carried out using various buffers and conditions to determine the optimal protocol for BMP-6 quantification. Results are currently being analyzed.

Table 1: Fluoroscopy images of tibia osteotomy and of gene delivery to fracture sites		
Pig number	**Osteotomy**	**Gene delivery session**

7228		
7252		
7266		

7270		
7517		
7664		

7720		
8175		

- **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, we intend to focus on Specific Aim 3: “Conduct a GLP toxicology study using the proposed therapy to treat critical-size bone fractures”. We will evaluate the effect of three different doses of SonoHeal’s injectable component at three different time points post-delivery on bone formation, toxicology and biodistribution in minipigs.

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?**
      An option agreement was signed between Cedars-Sinai Medical Center that owns the intellectual properties related to SonoHeal, and a startup company called GamlaStem Medical Inc. that aims to commercialize the use of SonoHeal.

    - **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**
      Nothing to report.

    - **Actual or anticipated problems or delays and actions or plans to resolve them**
      A delay in the initiation of the GLP study (Aim 3) was caused due to the extended time it took to obtain all animal protocol approvals. However, we do not expect this to affect the general time to achieve the milestones within the lifetime of the project.

    - **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
An option agreement was signed between Cedars-Sinai Medical Center and GamlaStem Medical Inc.

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://orcid.org/0000-0003-3857-5531	https://www2.scopus.com/authid/detail.uri?authorId=6602611025	https://www2.scopus.com/authid/detail.uri?authorId=12545883000
Nearest person month worked:	8	8	8	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?**
     A previously pending grant is now active:
     R01EB026094 (Gazit) 05/01/2018– 01/31/2022 2.4 calendar
     NIH/NIBIB
     Ultrasound-guided DNA delivery for regenerative medicine
     We proposed the development of an integrated system for image-guided transfection incorporating two unique transducers and associated signal processing that will facilitate successful in vivo gene delivery. We hypothesize that image-guided cavitation based on multi-frequency ultrasound (US) arrays will result in efficient critical-size fracture healing or tendon/ligament graft osteointegration.
     Specific Aims: Aim 1: Engineer and test image-guided transfection for critical-size bone fractures. Goal: 1A) Develop transducer and software to sweep the US beam within a 3D volume while mapping and controlling cavitation activity. Goal: 1B) Test the efficacy of fracture healing in a pig critical-size fracture model. Aim 2: Engineer and test image-guided transfection customized for ligament bone tunnels. Goal: 2A) Develop transducer and software to direct the US beam within a bone tunnel while mapping and controlling cavitation activity. 2B) Test the efficacy of enhanced repair in a pig ACL reconstruction model.

Role: Dan Gazit – PI (2.4 calendar), Gadi Pelled – co investigator (2.4 calendar), Zulma Gazit – co-investigator (2.4 calendar).
Overlap: None.
Contact: Randy King, Ph.D.
National Institute of Biomedical Imaging and Bioengineering (NIBIB)
6707 Democracy Boulevard, Suite 200 MSC 5477
Bethesda, MD 20892

- **What other organizations were involved as partners?**
  - **Organization Name:** 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 study was 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 RNA extraction assays under a service agreement with Covance Laboratories.

8. Special Reporting Requirements

None.

9. Appendices

None.