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CONTRACTING ORGANIZATION: University of Pittsburgh

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Juan Taboas, PhD

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5f. WORK UNIT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Effective treatment of war wounds in the forward combat setting and primary care facilities remains a significant challenge facing the military medical community. Battle wounds often necessitate early irrigation, debridement, and antibiotic therapy at the far-forward hospitals. Approximately 20% of battlefield wounds are bone fracture and loss at the extremities. However, antibiotic resistance remains a significant problem at primary military medical facilities, occurring in approximately 15% of surgical patients. The goal of this study is to developed a single-dose, injectable, prolonged nitric oxide (NO*) hydrogel-delivery system to treat deep wound infection and promote tissue regeneration. We hypotheses that (1) device formulations that release as low as an average of 20 μM/h NO* (approximately 6 nmol/h/cm2) for at least 18 hours will be bactericidal and biocompatible to mammalian cells in vitro and that (2) a total NO* dose of 30 μmoles/cm2 in the 5-mm defect will be bactericidal and promote bone regeneration in vivo. The specific aims of the project are to (1) evaluate the NO release, bactericidal efficacy, and biocompatibility of S-nitrosoglutathione (GSNO), short-term delivery microparticle (mSNO-MP), and long-term delivery microparticle (pSNO-MP) devices in vitro and (2) determine the efficacy in clearing infection (bactericidal) and in regenerating bone of hydrogels with GSNO, mSNO-MP, and pSNO-MP donors injected into infected femoral segmental bone defects in rats.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction

The long-term goal of this work is to drive clinical translation of a novel therapy for bone regeneration in infected limb injuries. Effective treatment of war wounds in the forward combat setting and primary care facilities remains a significant challenge facing the military medical community. Battle wounds often necessitate early irrigation, debridement, and antibiotic therapy at the far-forward hospitals. Approximately 20% of battlefield wounds are bone fracture and loss at the extremities. However, antibiotic resistance remains a significant problem at primary military medical facilities, occurring in approximately 15% of surgical patients. The microbial burden of acute and chronic wounds impairs healing and leads to infection-related complications such as sepsis, amputation, multi-organ failure and death. *The two critical problems are that no treatment exists to impede infection at the battlefield and no antibiotic cocktail exists with broad spectrum antimicrobial efficacy in infected wounds, particularly one that is efficacious against bacterial biofilms.* The goal of this study is to developed a single-dose, injectable, prolonged nitric oxide (NO*) hydrogel-delivery system to treat deep wound infection and promote tissue regeneration. NO* is a natural antimicrobial produced by the human immune system. It has broad-spectrum antimicrobial action against numerous microbial components. NO* is also known to enhance bone regeneration. However, NO* systems have not been developed for deep tissue applications. We have developed several NO* donors, particularly silicate microparticles, that are efficient carriers capable of delivering NO* over time, from brief delivery for a day to prolonged delivery for more than a week. We hypotheses that (1) device formulations that release as low as an average of 20 μM/h NO* (approximately 6 nmol/h/cm2) for at least 18 hours will be bactericidal and biocompatible to mammalian cells in vitro and that (2) a total NO* dose of 30 μmoles/cm2 in the 5-mm defect will be bactericidal and promote bone regeneration in vivo. The specific aims of the project are to (1) evaluate the NO release, bactericidal efficacy, and biocompatibility of S-nitrosoglutathione (GSNO), short-term delivery microparticle (mSNO-MP), and long-term delivery microparticle (pSNO-MP) devices in vitro and (2) determine the efficacy in clearing infection (bactericidal) and in regenerating bone of hydrogels with GSNO, mSNO-MP, and pSNO-MP donors injected into infected femoral segmental bone defects in rats.

2. Key words

Bone, bone marrow stem cell (BMSC), cartilage, fibrosis, fracture, gelatin, hydrogel, immunomodulation, infection, Methicillin resistant Staphylococcus aureus (MRSA), microparticles, nitric oxide (NO*), nitrosylated (3-mercaptopropyl) trimethoxysilane microparticle (mSNO-MP), nitrosylated D-penicillamine (S-nitroso-penicillamine) microparticle (pSNO-MP), Pseudomonas aeruginosa (P. aeruginosa) S-Nitrosoglutathione (GSNO), segmental defect, rat

3. Summary/Specific Aims and Accomplishments

What were the major goals of the project?

The Aims of the project are:

1. Evaluate the NO* release, bactericidal efficacy, and biocompatibility of S-nitrosoglutathione (GSNO), short-term delivery microparticle (mSNO-MP), and long-term delivery microparticle (pSNO-MP) devices in vitro
   1.1. (Sub-aim) Manufacture the NO* donors and hydrogel carrier system
2. Determine the efficacy in clearing infection (bactericidal) and in regenerating bone of hydrogels with GSNO, mSNO-MP, and pSNO-MP donors injected into infected femoral segmental bone defects in rats
   2.1. Sub-aim) Profile the leukocyte milieu in regenerate tissue via flow cytometry

The goals to accomplish are:

1. Provisional patent application
2. Publication on bactericidal efficacy in vitro of different $NO^{\cdot}$ donor formulations
3. Publication on NO* donor effects on human stem cell phenotype
4. Final patent application on device including bone outcomes

5. Advertisement of technology on the University of Pittsburgh's Office of Innovation and Technology Transfer website
6. Publication of device effects on MRSA infection and bone repair
7. Publication of device effects on immune response and bone repair
8. Pre-request for device designation by the FDA
9. Application to Coulter Foundation for preclinical study in pig

The statement of work with listing of completion and a discussion of the progress to date follow.

Values in Months, e.g. 0-3 = over 3 months stating first month	**Overall Timeline**	**Pittsburgh**	**Einstein**	**Zylö**	**USAISR**	**Fraction Completed**
Major Task 1: Fabricate the device formulations	1-18					
Subtask 1.1: Synthesize GSNO and SNO-MPs for Aim 1 and Aim 2 4 times each year				0, 6, 12, 18		30%
Subtask 1.2: Purify injectable hydrogel precursors and quality check for Aim 1 and Aim 2 2 times each year		1-3, 12-15				30%
Specific Aim 1: Evaluate $NO^{\cdot}$ release, bactericidal efficacy, and biocompatibility of the devices	1-12					
Major Task 2: Characterize $NO^{\cdot}$ release and donor degradation	1-4					
Subtask 2.1: Determine $NO^{\cdot}$ release from GSNO, mSNO-MP, and pSNO-MP devices				1-4		25%
Subtask 2.2: Image mSNO-MP and pSNO-MP degradation		1-4				0%
Major Task 3: Determine bacterial MIC and MBC of device formulations on MRSA and P. aeruginosa	1-6					
Subtask 3.1: MIC and MBC in planktonic growth			1-6			25%
Subtask 3.2: MIC and MBC in biofilms			1-6			0%
Major Task 4: Determine device formulation effects on human MSC viability and phenotype	1-12					
Subtask 4.1: Cytotoxicity and Proliferation		1-9				10%

Subtask 4.2: Phenotype characterization via PCR		3-12				0%
Milestone #1: Provisional patent application submission on device	12					
Milestone #2: Publication on bactericidal efficacy in vitro of different NO˙ donor formulations	13					
Milestone #3: Publication on NO˙ effects on MSC phenotype	15					
Specific Aim 2: Determine the bactericidal and bone regenerative effects of device formulations in infected rat segmental defect model	5-24					
Major Task 5: Culture bacteria for implantation		5-22				
Major Task 6: Perform the rat femoral segmental defect surgeries ± MRSA infection	6-22					
Subtask 6.1: Seek animal protocol approval by the Pittsburgh DLAR and the USAMRMC Animal Care and Use Review Office (ACURO)		2-4			1-6	50%
Subtask 6.2: Short-term evaluation of bacterial load and bactericidal efficacy of device formulations (infected wounds)		6-12 (60 animals)				0%
Subtask 6.3: Short-term evaluation of immunomodulatory effects of device formulations (uninfected wounds)					12-18 (60 animals)	0%
Subtask 6.4: Long-term evaluation of bone regeneration by the device formulations (infected and uninfected wounds)		12-22 (100 animals)				0%
Major Task 7: Determine bacterial load after treatment with device formulations (see subtask 7.2 for corresponding surgery)			6-13			
Major Task 8: Profile the leukocyte milieu in regenerate tissue via flow cytometry (see subtask 7.3)			12-19			

Major Task 9: Quantify bone regeneration (see subtask 7.4)		12-24				
Subtask 9.1: Analyze bone regeneration over time with in vivo μCT Imaging		12-22				0%
Subtask 9.2: Analyze final regenerate bone via high resolution μCT Imaging		13-23				0%
Subtask 9.3: Execute histological evaluation of the regenerate tissue composition (cell and tissue types) via biochemical and immunohistochemical stains		13-24				0%
Milestone #4: Final patent application on device including regeneration outcomes		24				
Milestone #5: Advertisement of technology on Pittsburgh's Innovation Institute website		24				
Milestone #6: Publication of device formulation effects on MRSA infection and bone regeneration	26					
Milestone #7: Pre-request for device designation with the FDA		26				
Milestone #8: Publication of device formulation effects on leukocyte infiltrate and bone regeneration in uninfected wounds	28					
Milestone #9: Application to Coulter Foundation to perform GMP large animal pilot study		30				

What was accomplished under these goals?

Major Task 1: Fabrication of device formulations

This task is in support of Aims 1 and 2, to manufacture the different NO* donors and hydrogel components.

Regarding Subtask 1.1, we synthesized GSNO and mSNO-MP donors for work on Aim 1 this year. We modified our synthesis protocols to adjust for desired pH of donors to match culture conditions. Further, based on results of Major Task 2 below, we adjusted the protocol for mSNO-MP to maximize NO* retention during washing microparticle washing steps. We further worked on the novel chemistry and particle synthesis of pSNO-MP. The particles are larger than mSNO and we are working to diminish their size and optimize NO* loading. We used these initial particles for Task 2 below, but will continue optimizing their formulation.

Regarding Subtask 1.2, we are using a commercially available collagen hydrogel as carrier.

Major Task 2: Characterize NO˙ release and donor degradation

Characterization of the donors is a major quality control and specification need of Specific Aims 1 and 2.

Regarding Subtask 1, we determined that GSNO has a burst release of NO* immediately upon dilution in buffered medium. We further determined that GSNO has a prolonged stable release of NO* in buffered medium. mSNO-MP show a faster delivery of NO*.

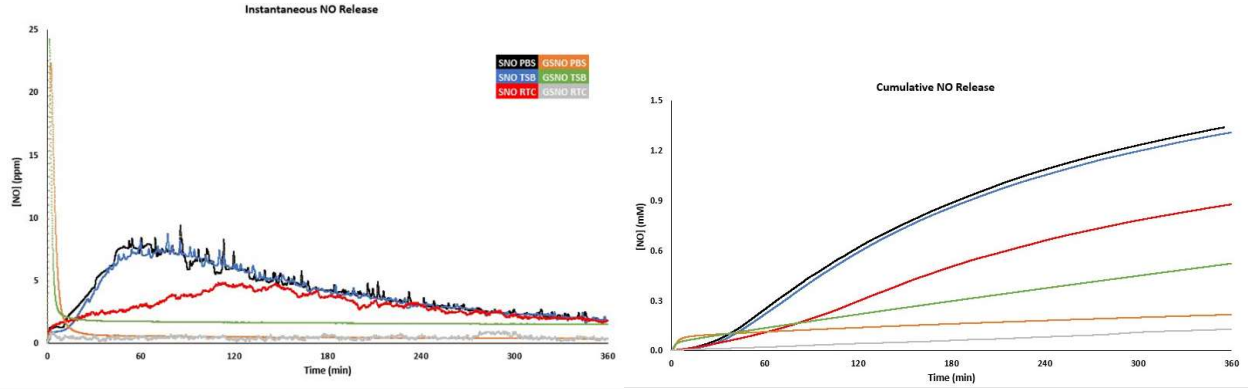

Figure 1: NO* release profiles from GSNO and mSNO-MP (SNO). (Left) Addition of GSNO to phosphate buffered saline (solvent for hydrogel, PBS) and tryptic soy broth (medium for planktonic culture of bacteria, TSB) induce a burst release of NO* within the first 5 minutes of dilution. No burst release is apparent when GSNO is added to the hydrogel (rat tail collagen at 3% w/v, RTC), but prolonged release is evident. mSNO-MP is a more stable NO* delivery vehicle than GNSO, in that it does not show a burst release upon addition to PBS and TSB. However, the mSNO-MP in RTC hydrogel shows a slower NO* release out to 2-hours, at which time instantaneous release begins to match that of mSNO-MP in the other buffers. Taken together, the instantaneous release results suggest that NO* partitions into the hydrogel (potentially reacting with primary amines). For GSNO in hydrogel, the burst NO* release quickly saturates partition sites on the hydrogel and NO* release is apparent from time zero. For mSNO-MP in hydrogel, saturation of partition sites on the hydrogel does not occur until 2-hours. (Right) The difference in mSNO-MP curves in PBS and TSB (blue and black curves) from that in hydrogel (RTC, red) after 2-hours plateaus, indicating saturation and the amount of NO* release required to saturate the hydrogel, approximately 3.5mM. However, this amount is much greater than the burst release recorded for GSNO, indicating that we are unable to fully quantify the burst. The NO*burst from GSNO must be greater than measured to saturate the same hydrogel and allow NO* release. The sample must be placed in the detector after dilution, and some NO* may escape during this step.

Regarding Subtask 2, in progress.

Major Task 3: Determine bacterial MIC and MBC of device formulations on MRSA and P. aeruginosa

This task is part of Aim 1. It focuses on a does response study to determine optimal concentration of NO* donors for bacteriostatic and bactericidal use.

Regarding Subtask 3.1, we have executed a dose response study of GSNO and mSNO-MP delivered to MRSA and *P. aeruginosa* in planktonic conditions

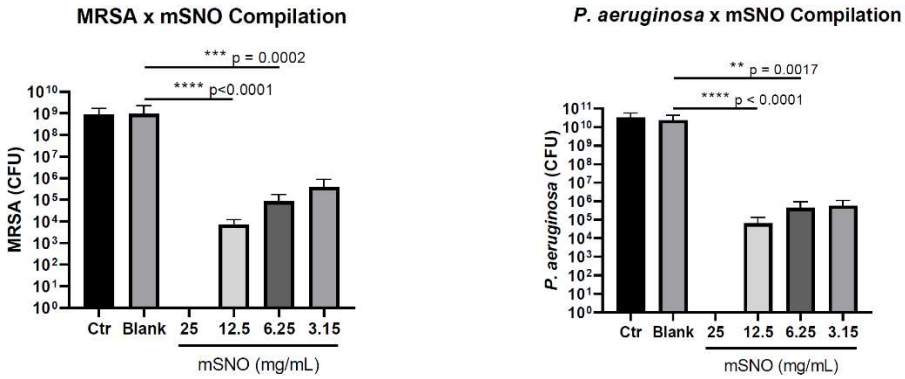

Figure 2: Response of MRSA and P. aeruginosa to the mSNO-MP. The bacteria display a clear dose response to the mSNO-MP with a critical threshold for bactericidal efficacy.

Regarding Subtask 3.2, biofilm assays are schedule for this Fall 2022.

Major Task 4: Determine device formulation effects on human MSC viability and phenotype

This task evaluates if the MIC and MBC doses are cytocompatible on human BMSCS, because these are one of the cell pools that lead to regeneration in the segmental defects. The aim further investigates the effect of NO* delivery on the phenotype of the cells, which will assist interpretation of the in vivo results of Aim 2.

Regarding Subtask 4.1, we have performed the cytotoxicity assays with GSNO.

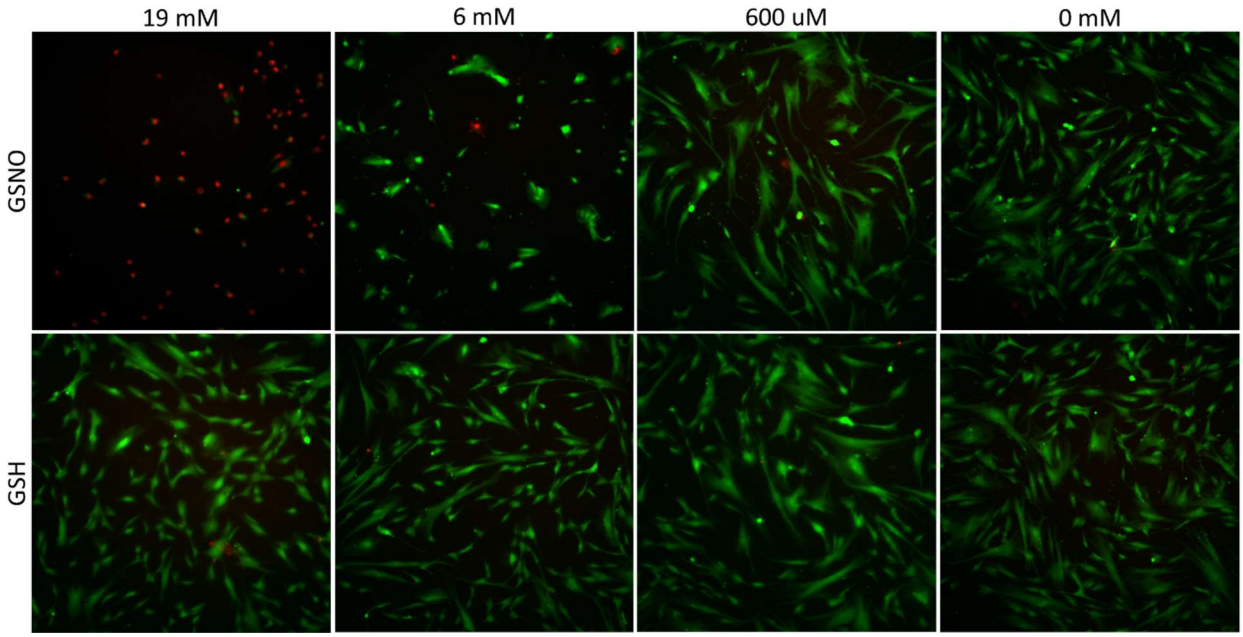

Figure 3: Viability of human BMSC at 24 hours post-treatment with nitrosylated glutathione (GSNO) versus reduced glutathione (GSH, control un-nitrosylated). Doses in the mM range impact cell viability, with 6 mM causing a altered cellular morphology and detachment. The higher dose is cytotoxic early during culture, such that cells do not detach). The GSH controls show no effect at matching molar doses.

Regarding Subtask 4.2, scheduled for initiation in Fall 2022.

Regarding Milestone #1: Invention not disclosed at this time pending more data.

Regarding Milestone #2: Pending data

Regarding Milestone #3: Pending data

Major Task 5: Culture bacteria for implantation

This task is performed as needed, to maintain bacterial stock for Aims 1 and 2

Major Task 6: Perform the rat femoral segmental defect surgeries ± MRSA infection

This task involves all the animal approval and treatment work (housing, operation).

Regarding Subtask 6.1, we have received IACUC and ACURO approval for the animal surgeries at the university of Pittsburgh site, but are awaiting approvals at the USAISR site. We have performed practice surgeries on cadaveric rats. In these, we injected radiopaque hydrogel to verify delivery into the boney defect. The hydrogel did not extrude past the defect.

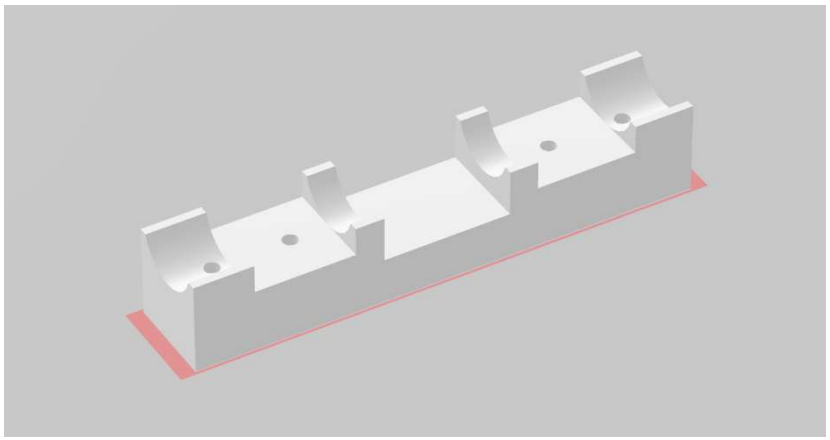

Figure 4: We have revised the design of our femoral segmental defect fixation device to accommodate the smaller size of the Wistar rats. We shortened the length and thickness, and relocated the screw holes to accommodate the design change. The curved mounts are placed on the femur. We have 30 fixation plates in hand.

Regarding Subtask 6.2, surgeries for testing bactericidal efficacy short-term, scheduled for Fall 2022

Regarding Subtask 6.3, surgeries for testing immunomodulatory effects short-term, pending USAISR ACURO

Regarding Subtask 6.4, surgeries for testing long-term efficacy in bone regeneration, scheduled for Spring 2023

Major Task 7: Determine bacterial load after treatment with device formulations

Pending animal surgeries.

Major Task 8: Profile the leukocyte milieu in regenerate tissue via flow cytometry

Pending animal surgeries.

Major Task 9: Quantify bone regeneration

Pending animal surgeries

Regarding Subtask 9.1, 9.2, 9.3, pending surgeries

What opportunities for training and professional development has the project provided?

1. One research technician was trained in numerous techniques needed in the project (scaffold fabrication, material modification)
2. One postdoctoral fellow was trained in bacterial cultures and relevant MBC and MIC assays
3. One graduate student was trained in mammalian cell culture and proliferation and viability assays.

How were the results disseminated to communities of interest?

1. "Nothing to Report."

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

In the next grant year, we will undertake significant progress in the project as our new collaborators at the USAISR come online. Regarding major task 1, we will have completed the design of the pSNO-MP and characterizations of all three donors. Regarding Aim 1, we will complete the bacteria and BMSC dose response studies. Regarding Aim 2, we will make headway in the non-infected animal experiments at the USAISR and Pittsburgh. We will begin to probe the leukocyte response to the different donor compositions in vivo. We will be performing treatments with MRSA infection in the third quarter of the second grant year.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

1. "Nothing to Report."

What was the impact on other disciplines?

1. "Nothing to Report."

What was the impact on technology transfer?

1. "Nothing to Report."

What was the impact on society beyond science and technology?

1. "Nothing to Report."

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

"Nothing to Report."

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

We encountered significant delay in the project this year for the following reasons:

1. Personnel changes at the USAISR site. The leadership at the USAISR underwent significant change with the retirement of our key collaborator, Dr. Erik Weitzel. Our collaboration was subsumed by Maj Casey Sabbag, MD, under leadership of the USAISR by Col Joseph Alderete Jr, MD. Coordinating the transition caused delay due to Col Alderete's deployment.
2. Experiments with NO* had to be repeated, because of particles manufacturing out of specification
3.

Changes that had a significant impact on expenditures

1. "Nothing to Report".

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

1. "Nothing to report"

6. PRODUCTS: List any products resulting from the project during the reporting period. Examples of products include:

Publications, conference papers, and presentations

1. "Nothing to Report."

Books or other non-periodical, one-time publications.

1. "Nothing to Report."

Other publications, conference papers, and presentations.

1. "Nothing to Report."

Website(s) or other Internet site(s)

1. "Nothing to Report."

Technologies or techniques

1. "Nothing to Report."

Inventions, patent applications, and/or licenses

1. "Nothing to Report."

Other Products

1. "Nothing to Report."

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Project Role	Research Identifier	Person Months Worked	Contribution to Project	Funding Support
Joseph Alderete	Co-I		0	Clinical guidance on osteopathology and interpretation of data	USAISR
Alejandro Almarza	Co-I		1	Mechanical testing for biomaterials and tissues. Animal surgeries, data acquisition and interpretation.	
Jennifer Cox	Laboratory Administrator		4	Management of sub-award laboratory, supplies ordering, schedule coordination.	USAISR
Andrew Dragnaski	Co-I		3	Microparticle synthesis and materials characterization	Zylo Therap eutics
Erik Hegeman	Resident Fellowship		1	Preparation of animal protocol at USAISR	USAISR
Gabrielle Lorenz	Graduate Student		2	Aim 1 mammalian cell assays	
Ingrid McNamara	Laboratory Administrator		1	Management of principal-award laboratory, hydrogel preparation, animal surgeries, histology.	
Joshua Nosanchuk	Co-I		1	Bacterial assays and immunological analysis of host/tissue response	
Casey Sabbag	Co-I		1	Sub-award PI. Animal surgeries, data acquisition, and interpretation. Foster collaboration with sub-award	
Juan Taboas	PI		3	Preparation of animal protocol. Development of biomaterials and devices. Animal surgeries, data acquisition and interpretation. Overall management of project	
Erik Weitzel	Co-I		0	Sub-award PI. Animal surgeries, data acquisition, and interpretation. Foster collaboration with sub-award	USAISR
Daniel Zamith	Postdoctoral Fellow		6	Aim 1 bacterial assays	

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

Funding Agency	NIAMS, NIH
Grant Number:	1R21 DE032478-01
Title of Grant:	Identification of selective inhibitors of PTH-receptor for Jansen's metaphyseal chondrodysplasia
Principal Investigator	Juan Taboas and Jean-Pierre Vilardaga
Taboas Role on Grant	Co-PI (2 co-PIs)
Years Inclusive	9/01/2022-8/31/2024
Percent Effort	10 %

What other organizations were involved as partners?

1. Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS COLLABORATIVE AWARDS:

1. "Nothing to report"

QUAD CHARTS:

1. Attached

9. APPENDICES:

None