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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

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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˙ 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 achieved two goals:

1. We optimized neutralization of our mSNO-MPs and repeated antimicrobial and BMSCs assays. We detected cytotoxicity in the BMSCs due to insufficient pH neutralization after nitrosylation.
2. We validated that our in-house synthesized GSNO does not contain residual salts that impair cell viability. We did so by depleting the NO* from GSNO using UV-A illumination and showing that this inactivated GSNO (GS*) did not impact cell viability (Live/Dead) nor aerobic metabolism (MTS assay) compared to glutathione (GS) and control salt solutions (HBSS)
3. We successfully fabricated our prolonged release NO* donor, pSNO-MP. The particle size is larger than mSNO-MP and rougher, as the microparticle (MP) is produced from a ground stock. We developed a protocol for optimum activation of the pSNO-MPs (loading of the MP with NO*). Our initial protocol contained residual chemicals that impaired cell viability.

With these goals met, we have demonstrated the synthesis of all three of our NO* donors (GSNO, mSNO-MP, and pSNO-MP, **Figure 1**).

Figure 1. Appearance of NO* activated donors in solution.

Regarding subtask 1.2, we validate that our rat tail collagen hydrogel consistently forms gels between lots of commercial batches. We also investigated an alternate human derived source with clinical translation in mind and the potential to create stiffer gels with slower degradation, as it is provided in an 8% w/v solution as opposed to the 3% w/v of rat tail. However, this source proved less effected in gelation and with deleterious effect on cell adhesion (Figure 2).

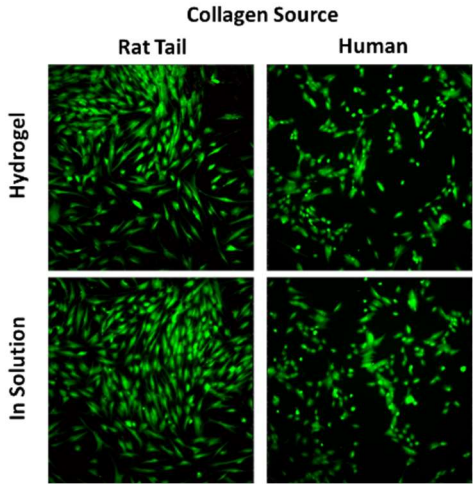

Figure 2. The human derived collagen does not form a hydrogel of adequate mechanical consistency for use as a carrier for the NO* donors compared to the rat tail-derived collagen. The cells were exposed to either the hydrogel made of these collagens (top row) or the neutralized collagen solution (supplemented into medium before gelation). Cultures were performed at 37C, 5% pCO2 and 20% pO2.

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 2.1, we will initiate characterization of NO* release from our pSNO-MP in the next award year. We previously determined the release kinetics of GSNO and mSNO-MP.

Regarding Subtask 2.2, in progress now that we have successfully synthesized pSNO-MP.

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

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

Regarding Subtask 3.1, we re-tested the antimicrobial effect of our mSNO-MPs on MRSA and Pa in planktonic conditions using equivalent NO* doses as our prior mSNO-MP data (Figure 3).

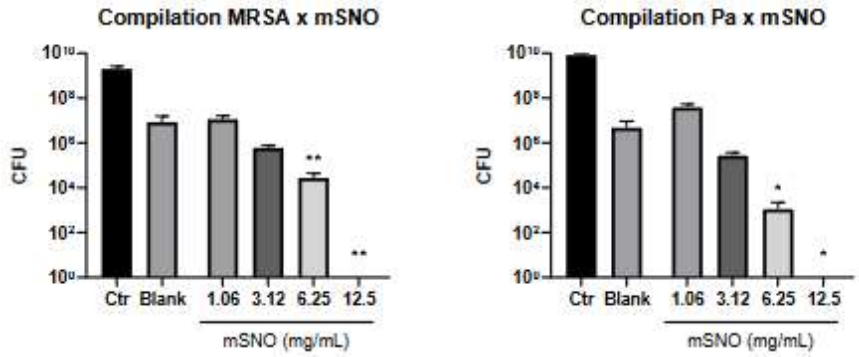

Figure 3. The dose for optimum microcidal activity of mSNO-MP was selected as 10 ug/ml. We defined the MBC (minimum bactericidal concentration) as the dose that induces a 4-fold change in CFU's (colony forming units). The dose of 6.25 mg/ml was the minimum achieving the desired MBC.

Regarding Subtask 3.2, biofilm assays are scheduled.

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 achieved several goals:

1. We determined that the transwell culture system as provided by the manufacturer was faulty. We experienced a series of aberrant results with our Live/Dead, MTS (mitochondrial respiration via the tetrazolium salt reduction), and Quant-iT PicoGreen (cell number via DNA quantification) assays. The Live/Dead showed patches of culture dishes where cells were not present at random locations within wells of technical replicates. The MTS and PicoGreen results were inconsistent across technical replicates. We determined that the transwells (contain the hydrogel with NO* donor) make contact with the baseplate well on which the cells are seeded (Figure 4). We created a polydimethylsiloxane (PDMS) gasket that fits on the baseplate to elevate the transwells. We subsequently repeated all experiments reported below.
2. We created a ring insert system to restrict seeding of cells to a circular area directly below the transwell (Figure 5). This eliminates gradient effects of the NO* diffusion. We validated that the insert system did not impact cell metabolism (Figure 6).
3. We investigated the dose effect of the mS-MP with and without NO* loading on the human BMSC morphology and viability (Figures 7, 8)
4. We investigated dose effect of the mS-MP with and without NO* loading on the human BMSC proliferation via the MTS assay (Figure 9). We identified variability in the multiplex assays (coupling Live/Dead, MTS, and PicoGreen) in the same wells and are optimizing assay conditions.
5. We began investigation the dose effect of nitrosylated and un-nitrosylated glutathione (GSNO and GS*, respectively) and our pS-MPs on the BMSC viability. We used a matched total NO* loading. For pS-MPs, no effect was observed in cell morphology across all doses (Figure 10). As noted in the last year report, the GSNO donor releases NO* rapidly compared to the mSNO-MPs. This led to cytotoxicity at all but the lowest doses (Figure 11). We are determining if a GSNO dose exists that is microcidal without cytotoxicity to mammalian cells. We previously explored doses with a total NO* loading lower than that of our mSNO-MPs. We will use this lower dose for our in vivo work, but this will preclude direct comparison to the mSNO-MPs and pSNO-MPs, as the total loading of NO* in GSNO will necessarily be lower.

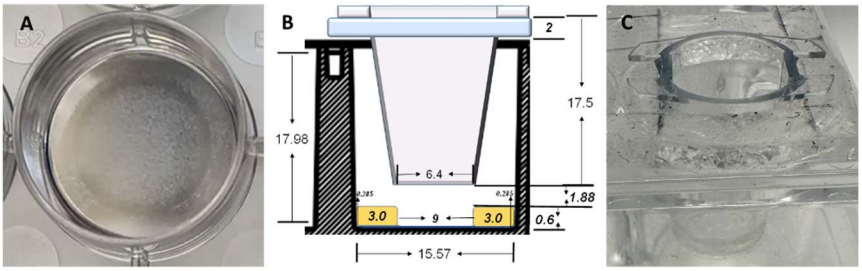

Figure 4. (A) The transwell dishes make contact with the baseplate. In some cases, a complete loss of cells bellow the cylindrical wall of the transwell is evident. (B) We designed a 2 mm PDMS gasket to raise the transwells. (C) The gasket in a dish. The dish lid fits slightly raised on the dish.

Figure 5. The PDMS inserts for controlled seeding of cells directly below the transwells.

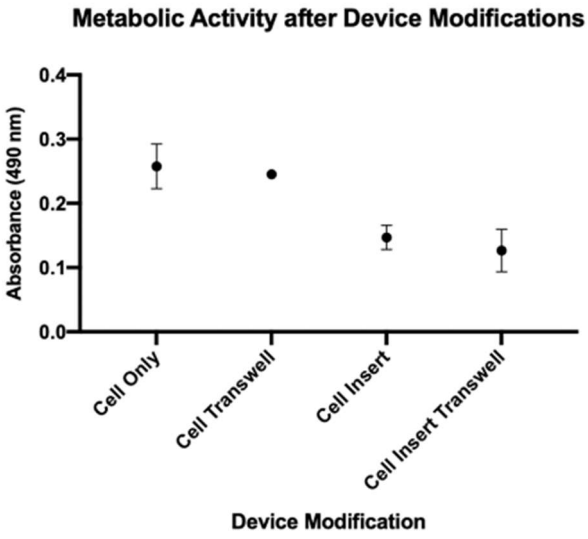

Figure 6. The transwell leads to a apparently slight (not statistically significant) decrease in MTS readings in both open wells and wells fitted with the PDMS. The inserts have no effect on the cells, other than restricting seeding of a smaller region of the well (ergo lower MT with insert than without).

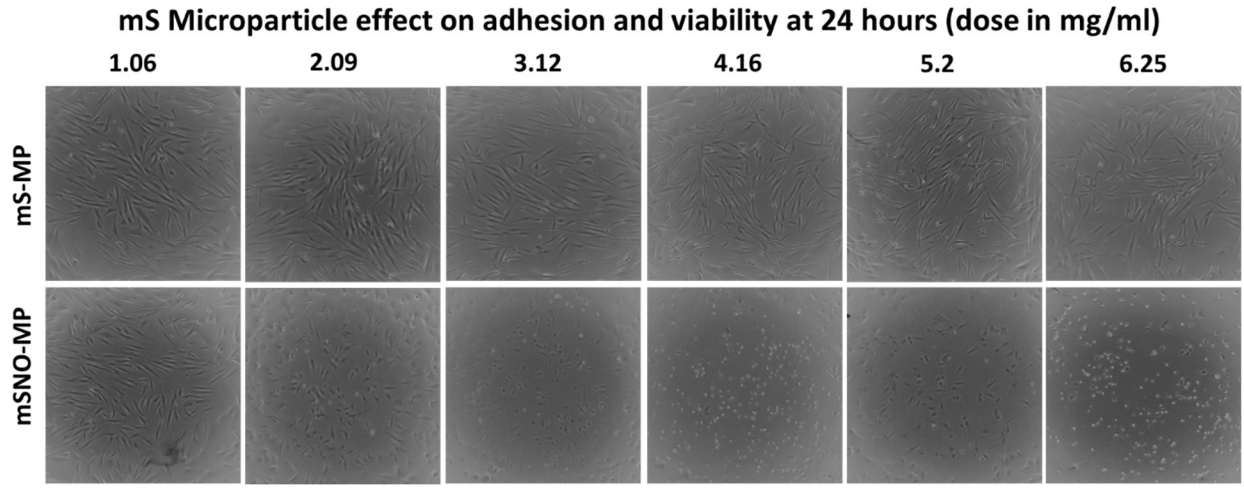

Figure 7. At 24 hours post-delivery, the mSNO-MPs show a dose effect on the spreading of human BMSC but no effect on the viability (Live/Dead assay, not shown).

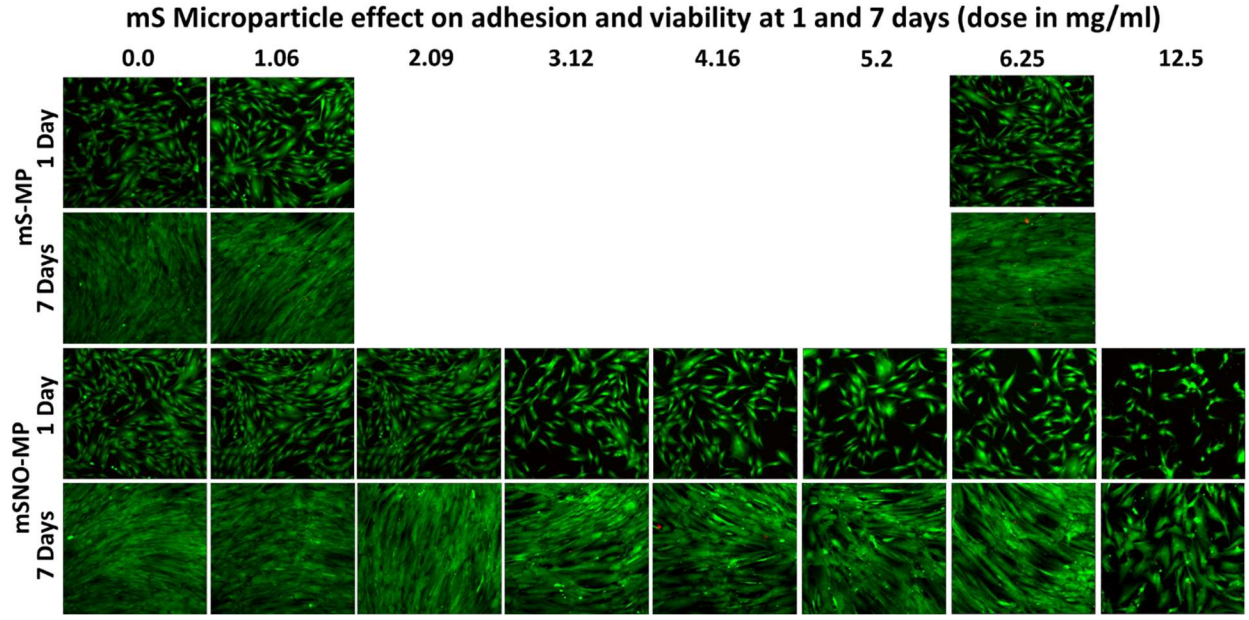

Figure 8. The mSNO-MP doses had no apparent effect on cell morphology until the 12.5 mg/ml dose.

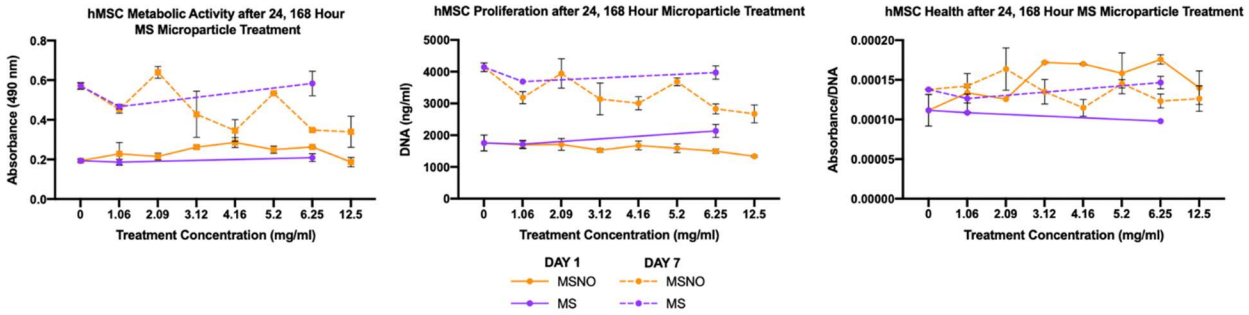

Figure 9. NO* delivery via mSNO-MPs appears to suppress cell proliferation in normoxic (20% pO2) cultures over time. We are investigating the interaction with pO2 using an optimized multiplex assay with greater precision. The

control mS-MPs (without NO*) have no effect on respiratory metabolism (absorbance of MTS at 490nm) or proliferation (DNA)

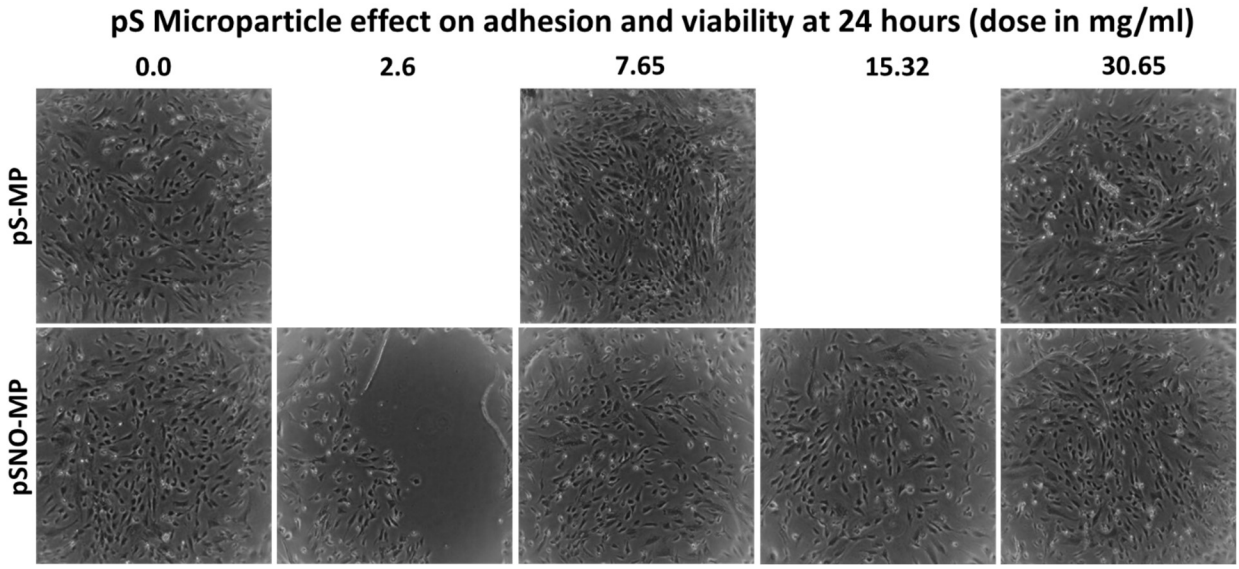

Figure 10. The pSNO-MPs show no effect on the BMSC adhesion and spreading, unlike the mSNO-MP at 12.5 mg/ml. Note, equivalent NO* loading was tested between pSNO-MPs and mSNO-MPs, e.g. the 30.65 mg/ml pSNO-MP dose is equivalent to the 12.5 mg/ml pSNO-MP dose in total NO* loading.

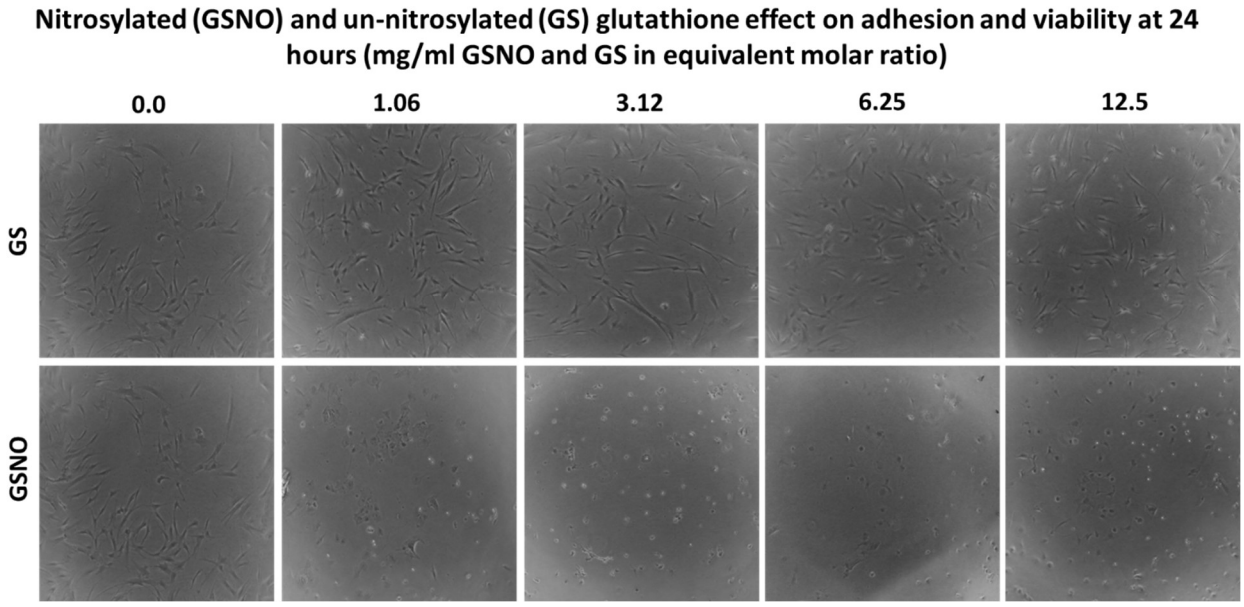

Figure 11. Doses of GSNO in the mg range are cytotoxic to human BMSCs. Cells are detached and unhealthy at the lowest equivalent dose of 1.06 mg/ml. Equivalent molar dose of GS shows effects at high doses.

Regarding Subtask 4.2, we performed analysis of the mSNO-MP dose range on expression of stem cell-like markers in the human BMSCs of one donor (Figure 12). Gene expression was assayed at 1-day and 7-days treatment. mS-MPs were used as controls (same mass matched dose to mSNO-MP). Both mSNO-MPs and mS-MPs were carried in hydrogels within transwells placed above the cells in the tissue culture baseplate. Cells cultured on tissue culture plates without transwells served as untreated controls. NO* delivery was found to decrease expression of stemness markers Sox2, Oct 4, and Nanog. The results suggest that NO* may promote differentiation of BMSCs in vivo, as

evidenced by the decrease in stemness markers in the physiologic (5% pO2) cultures. We have planned further analysis for gene expression of stemness markers and control (reference) transcripts, and further biological replicates with human BMSCs from additional donors.

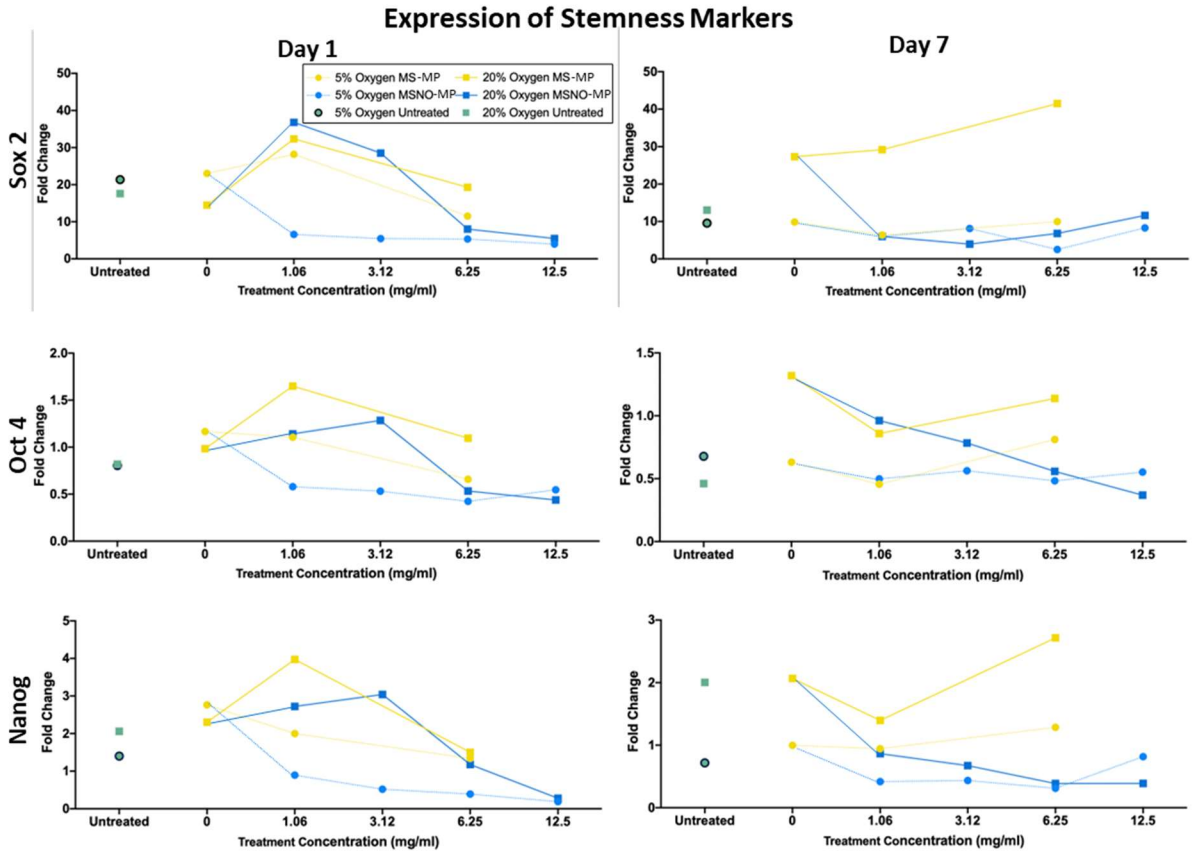

Figure 12. NO* delivery decreased expression of stemness markers Sox2, Oct 4, and Nanog, and oxygen tension of culture conditions interacted with NO* in regulating gene expression. *NO* effects*: In physiologic O2 cultures (5% pO2), NO* delivery significantly decreased Sox2 by approximately 5-fold at all mSNO-MP concentrations after 1-day exposure. In normoxic cultures (20% ambient pO2), only the 6.25 and 12.5 mg/ml doses showed a similar decrease in Sox2 at 1-day. Continued delivery of NO* led to a decrease in Sox2 by all mSNO-MP doses in normoxic cultures at 7-days to levels dissimilar from physiologic cultures. Expression patterns at 1-day were similar for Oct 4 and Nanog with similar magnitude of effect, e.g. 2.5-fold decrease in Oct 4 and 4.5-fold decrease in Nanog with mSNO-MPs in normoxic cultures. By 7-days, continued delivery of NO* led to a decrease in Nanog by all mSNO-MP doses in normoxic culture, as ween with Sox2. At 7-days 3.12 mg/ml dose of mSNO-MP approached that of higher dosed, but not the lower dose. *pO2 effects*: Oxygen tension effects were inconsistent across markers. Only Nanog showed a consistent decrease of expression over time with normoxia in untreated controls. Sox2 expression was also sensitive to oxygen tension, with approximately 1.5-fold greater expression in physiologic O2 at 1-day but decrease at 7-days. *Transwell system effects*: The transwell system (inserts with hydrogel and varying doses of particles) impacted gene expression, with mS-MPs decreasing markers in physiologic cultures but not normoxic cultures at 1-day treatment. This effect did not follow the pattern of marker expression by oxygen tension in untreated controls, precluding concern over transwell-limited oxygen diffusion as a convoluting regulator of gene expression.

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

Regarding Milestone #2: In preparation and pending data from characterization of NO* release from pSNO-MP.

Regarding Milestone #3: Pending execution of biological replicates

From the results of Major Tasks 1-4, we determined the optimal dose of mSNO-MP for microcidal and pro-regenerative effects was 10 mg/ml. This dose was subsequently used in the following tasks of our Specific Aim 2.

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 recently received ACURO approval for the USAISR site for the surgeries pertaining to sub-task 6.3. We previously reported IACUC and ACURO approval for the animal surgeries at the university of Pittsburgh site. We performed several surgeries at the Pittsburgh site pertaining to Subtasks 6.2, 6.3, and 6.4.

Regarding Subtask 6.2, we treated 3 animals with 10 mg/ml mSNO-MPs and 5 animals with 24.5 mg/ml pSNO-MPs (equivalent dose to the 10 mg/ml mSNO-MP). Half of the animals were infected with MRSA (2 for each NO* donor).

Regarding Subtask 6.3, surgeries for testing immunomodulatory effects at short-term time-points are pending facilities and personnel availability at the USAISR.

Regarding Subtask 6.4, we performed 10 surgeries without hardware failure, with 5 animals treated with hydrogel containing 10 mg/ml mSNO-MPs and 5 animals containing hydrogel alone. We also infected 2 animals with MRSA and treated with either mSNO-MP loaded hydrogel or hydrogel alone.

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

We have six samples awaiting quantification of bacterial load in the wound site pending arrival of a bone homogenizer. The homogenized tissue will be plated on agar and presence of bacteria identified. We tested efficacy of the mSNO-MP (deliver NO*) particles versus mS-MP (contain no NO*) at 10 ug/ml on MRSA contaminated wounds. Additional controls were wounds not experimentally contaminated with MRSA to identify potential iatrogenic infection.

We have performed tail vain blood draws of MRSA treated animal to determine risk of sepsis. No evidence was found (Figure 12).

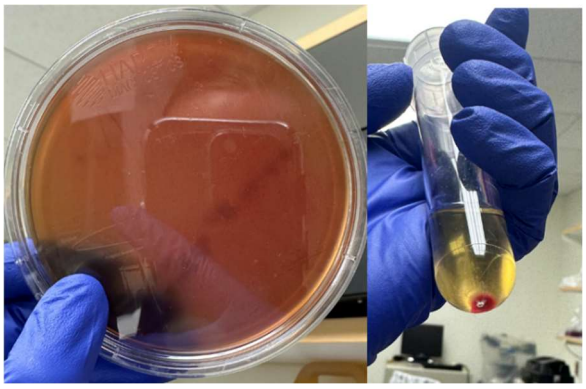

Figure 12. No evidence of MRSA has been found in circulating blood, indicating a low risk of sepsis in the experimental model. Presences of bacteria was detected by colonies on the plate and growth in broth.

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

We have been working to optimize our flow cytometry analysis using wound samples and control spleen tissue (Figure 13). We expect completion of protocol optimization by the new year.

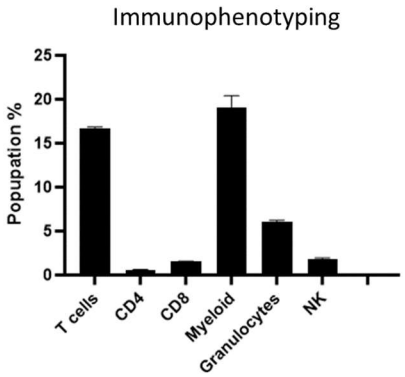

Figure 13. We tested our flow cytometry protocol for identification of the major leukocyte types. We are further optimizing the protocol for identification of T helper cell and macrophage phenotypes (Th1, Th2, Th17, M1, and M2).

Major Task 9: Quantify bone regeneration

Regarding Subtask 9.1, 9.2, 9.3, we have performed terminal uCT imaging of all animals and are processing samples for histological analysis. We have detected an anabolic effect of the 10 mg/ml mSNO-MP dose in the limited replicates at this time (Figure 14). Osteogenesis is evident in the NO* treated defect compared to controls. Full bridging does not occur over the 6 week time-point. Repeated measures over time with uCT was not possible due to equipment failure in the DLAR. We subsequently used alternate modalities, namely CT (low resolution at 110 um voxel) for uninfected animals and x-ray for MRSA treated animals. MRSA treated animals cannot be CT in our DLAR facilities because they cannot be removed from ABSL-2 enclosure to the large animal CT room.

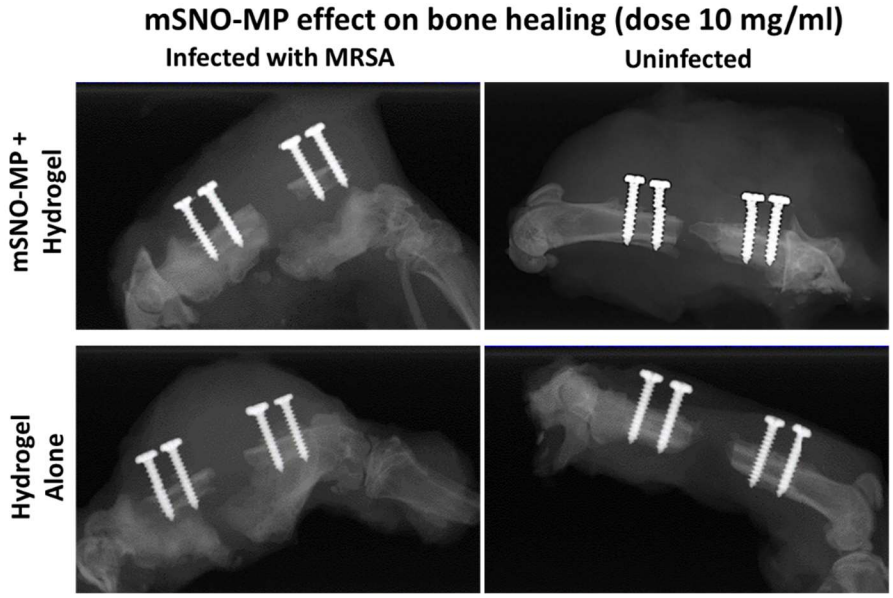

Figure 14. The mSNO-MPs promote regeneration in the critically sized defects in rats (Uninfected on right), but infection is unresolved leading to bone remodeling, lysis and hardware failure.

Regarding Milestone #4: Final patent application is pending Milestone # 1.

Regarding Milestone #5: Advertisement of technology is pending Milestone #1.

Regarding Milestone #6: Publication on MRSA and bone repair is pending biological replicates.

Regarding Milestone #7: Request for device designation is pending Milestone #4.

Regarding Milestone #8: Publication of immunomodulatory effects is pending biological replicates.

Regarding Milestone #9: Large GMP studies is pending Milestone #8.

From the results of Major Tasks 5-9, we determined the 10 mg/ml mSNO-MP does promotes repair of the critically sized femoral defects in rats. Further, MRSA infection causes bone lysis and remodeling, and decreases stability of the plating hardware.

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

1. Our graduate student was trained in the literature of NO* delivery on skeletal regeneration, NO* effects on skeletal development, stem cell biology, and RT-qPCR analysis. The successfully passed their qualifying exam based on this project and are now preparing for their candidacy exam. They were awarded an internal fellowship (Berenfield Graduate Fellowship in Cardiovascular Bioengineering) for a proposal directly related to the DoD project.

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?

We will initiate characterization of NO* release from our pSNO-MP in the next award year, and analyze the degradation of both the mSNO-MP and pSNO-MP donors. We will complete the manuscript on bactericidal efficacy of the donors with this characterization data. We will initiate surgeries at the USAISR site and evaluate the short-term modulatory effects with our two MP donors. We will begin quantification of bacterial load in the wounds treated with mSNO-MPs and perform surgeries to do the same with our pSNO-MP donor. We also plan to add histologic staining of bacteria in biopsies of the defects. We will continue the long-term surgeries evaluating mSNO-MP effects on bone healing, and will perform the same with the pSNO-donor. Our bacterial contamination titer is high compared to the literature and expected contamination in acute wounds at forward battlefields. We will therefore test lower titers, combination of donors (namely addition of the rapid release donor GSNO with mS-MP and pS-MP), and co-delivery of antibiotic in the infected groups to drive bacteria eradication. We plan to file a technology disclosure in coordination among the multiple worksite entities on the antimicrobial and regenerative effects, and submit an abstract on the work to the MHSRS 2024.

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. Major Tasks 2, 3, and 4: Development of manufacturing processed delayed initiation of characterization of pSNO-MP and testing their antimicrobial and BMSC effects.
2. Major Tasks 3 and 4: Experiments with NO* had to be repeated, because of defects in the transwell systems of commercial vendors. We have resolved the problem with a PDMS gasket as discussed above.
3. Major Task 8: The USAISR site received ACURO approval this year for the surgeries of tissues for flow-cytometry. They are slated to begin surgeries at the end of October. To assure completion of the project in the NCE year, we plan to also perform Task 9 surgeries at the USAISR site in addition to the Pittsburgh site.
4. Major Task 9: Surgical hardware failed due to refractory behavior of the animals during repeated measures CT and x-ray. The animals would kick during restraint for injection of the anesthetic (ketamine and xylazine), which caused dislocation of the plate screws (most often at the proximal femur site). We plan to purchase our own isofluorane anesthesia device so that handling of animals is minimized during these procedures (no injection needed).
5. Regarding Subtask 9.1: The in vivo uCT source failed and the DLAR has no funds to repair. We do not have sufficient funds in this project for the $35,000 repair. Thus, we have switched to using an in vivo CT (lower resolution). However, we cannot perform CTs of the MRSA infected animals on this system because transport of animals beyond the ABSL2 barrier is not permitted. For the MRSA animals, we are using x-rays over time.

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 Therapeutics
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	
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. Albert Einstein College of Medicine
2. USAISR
3. Zylo Therapeutics

8. SPECIAL REPORTING REQUIREMENTS COLLABORATIVE AWARDS:

1. "Nothing to report"

QUAD CHARTS:

9. APPENDICES:

None