Nitric Oxide Synergizes with TGF-β1 in Healing of Segmental Bone Defects

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INTRODUCTION. The limited early treatment of open bone fractures in low resource settings (LRSs) increases infection and chronic non-union rates (42% and 6-12x, respectively). LRSs include Health Professional Shortage Areas (HPSAs), military battlefields, and low income countries (LICs). Nearly 20% of Americans live in HPSAs, where rural hospitals report higher rates of antimicrobial resistant (AMR) infections that are 2–3x more costly to treat. On the battlefield, contaminated bone wounds account for 20% of injuries. In LICs, which account for 80% of the world's 455 million fractures per year, care is often delayed as patients travel an average of four days to the nearest hospital and is unaffordable as 80% of the population lives below the poverty line. These realities underscore the need for a low-cost and shelf-stable therapeutic to prevent AMR infections and accelerate bone healing in LRSs.

Our team has previously reported the development of an antimicrobial therapeutic that, unlike antibiotics, is inexpensive and has no known bacterial resistance. It is a spreadable/injectable hydrogel containing transforming growth factor beta 1 (TGF-β1) and microparticles (MSNO) that deliver nitric oxide ($NO^{\cdot}$). TGF-β1 is sequestered in bone and is a key player in bone homeostasis. Following fracture, $NO^{\cdot}$ is rapidly produced by the immune system to clear infections. After this inflammatory phase, $NO^{\cdot}$ concentrations drop, promoting wound contraction, osteogenesis, and angiogenesis to support bone regeneration. Exogenous delivery of $NO^{\cdot}$ supports bone healing, enhancing callus size and fracture stability. Though the regenerative benefits of $NO^{\cdot}$ are clear, its translation is limited because endogenous $NO^{\cdot}$ has a short physiological half-life and rapid diffusion. Attempts to exogenously deliver $NO^{\cdot}$ are complicated by burst release, lack of prolonged release, and donor impurities that exert cytotoxic effects. Our therapeutic employs a new microparticle delivery system, MSNO (silica microparticles of mercaptoporopyl trimethoxysilane) that stabilizes and prolongs delivery of $NO^{\cdot}$ over three days. We showed it reduces the bacterial load in biofilms of methicillin-resistant S. aureus (an AMR) by 99% in vitro and significantly reduces the bacterial load of femoral bone defects inoculated with 500,000 CFU of MRSA in rats. However, our therapeutic currently relies on a rat tail-derived collagen hydrogel (RTC) as the scaffold and MSNO carrier, which poses translational concerns including antigenicity, slow gelation, impractical shipping requirements, and cost.

Herein, we study the effect of MSNO on the immune response and regenerative efficacy of our therapeutic formulated with two hydrogels, RTC and PEG-GEL. PEG-GEL is comprised of azide-functionalized gelatin crosslinked with a 4-arm polyethylene glycol functionalized with dibenzocyclooctyne. It sets within 5-10 minutes of mixing via a strain promoted azide-alkyne cycloaddition (SPAAC) chemistry, which is biorthogonal, mechanically tunable, and functionally versatile. Unlike RTC, which requires thermal activation and lacks chemical specificity, the PEG-GEL forms rapidly, enabling controlled crosslinking and preserving biologic function of small molecules and cells. We used co-cultures of human bone marrow stromal cells (BMSC) with MSNO laden hydrogels in basal and osteogenic medium to probe osteogenesis. We implanted the hydrogels and profiled the leukocyte infiltrate at 5 days post-implantation with flow cytometry and bone healing with -rays over time, and at 16 weeks with terminal μCT and histology.

METHODS: *Manufacturing*: The nitrosothiolated microparticles were fabricated using a sol-gel process (US20190365797A1). A 21.2 μM $NO^{\cdot}$ dose (10 mg/mL MSNO) previously determined to be microcidal and biocompatible, and 100 μg/ml of human TGF-β1 was incorporated into the hydrogels. RTC hydrogels were formed at 0.3% w/v and PEG-GEL at 2% w/v at a molar ratio of 1:12 PEG-GEL. *In vitro BMSC assays*: Donor-laden hydrogels were cast in 0.4 μm diameter pore transwells positioned 2 mm above the tissue culture plastic surface to model the proximity of $NO^{\cdot}$ delivery *in vivo*. BMSCs (passage 3, n=3) were seeded below and cultured under 5% (p05, mimicking wound) and 20% (p20, conventional culture) oxygen partial pressure. Transcripts indicative of differentiation state were analyzed with RT-qPCR at 24 hours and 1-week culture. *In vivo bone defect*: A unilateral 5.0 mm mid-diaphyseal segmental defect was created in the femurs of skeletally mature male Wistar rats (IACUC approved study). Male rats were used for consistency with the previous infection model that justified sole sex based on reduced immune function compared to females. A factorial assay was run to compare the effect of hydrogel and drug type with PBS-only injection and NO-free microparticles (MS) as controls. Biological replicates are 6 rats per treatment. The total rat number is 96 (48 for immune and 48 for regeneration). Each treatment was administered 24 hours post-surgery to mimic delayed clinical intervention. *Leukocyte profiling:* Defects were biopsied 5 days post-treatment, mechanically dissociated, and analyzed for granulocytes, macrophages, and T cell types by flow cytometry using the following antibody panel: CD45, CD3, CD4, CD8, CD161, CD11b/c, CD86, CD163, and His48. *Bone regeneration*: Healing was assessed using X-rays over time and terminal high-resolution (6 um voxel) micro-CT analysis and histomorphometry of serial sections.

RESULTS: *BMSC assays*: qPCR analysis demonstrated an $NO^{\cdot}$ dose-dependent reduction in markers of stemness (Sox2, Oct4) and chondrogenesis (aggrecan), while an increase in markers of osteogenesis (osteocalcin, alkaline phosphatase) that were greatest at 7 days (Figure 1). *In vivo bone defect:* Defects treated with MSNO and TGF-β1 in either RTC or PEG-GEL carriers demonstrated earlier and consistent bone formation, with at least 50% bridging achieved before 9 weeks. By 12 weeks, these groups achieved at least 80% bridging across the defect length, while controls showed no significant new bone formation (Figure 2). Flow cytometry revealed that RTC markedly increased $CD4^+$ and $CD8^+$ T cells while decreasing granulocytes relative to all treatment groups, whereas PEG-GEL slightly reduced $CD4^+/CD8^+$ T cells and increased granulocytes relative to RTC. Supplementation with MSNO or TGF-β1 suppressed both $CD4^+$ and $CD8^+$ T cell responses in either carrier. Notably, TGF-β1-treated groups consistently exhibited a low M1/M2 ratio along with suppressed $CD4^+$ T cells (Figure 3).

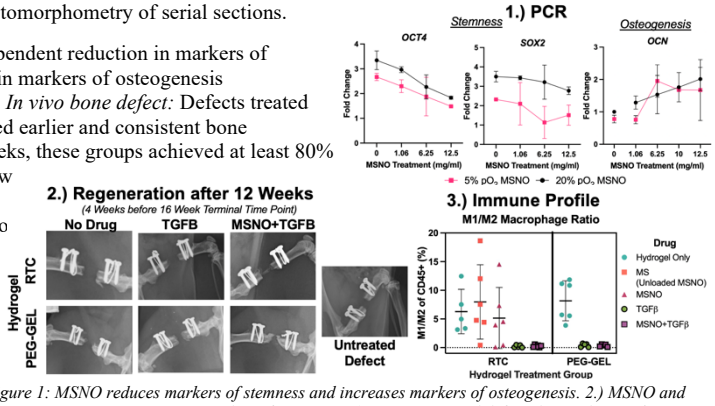

Figure 1: MSNO reduces markers of stemness and increases markers of osteogenesis. 2.) MSNO and TGF-B1 synergize to promote new bone formation. 3.) TGF-B1-treated defects have a low M1/M2 ratio.

DISCUSSION: The single dose MSNO hydrogel treatment may serve as an adjuvant to promote bone regeneration in compromised wounds. Our data supports prior works showing $NO^{\cdot}$ delivery accelerates osteogenesis. The results show a direct mechanism in promoting osteogenesis by BMSCs *in vitro*, potentially driving earlier bony bridging *in vivo*. $NO^{\cdot}$ synergized with TGF-β1yielding greater osteogenesis than either alone. Further, $NO^{\cdot}$'s mechanism seems to involve modulation of the leukocyte milieu. Though MSNO did not significantly alter the M1/M2 ratio alone or with TGF-β1, it did modulate the granulocyte compartment in PEG-GEL. Regarding hydrogel effects, they did not appreciably impact bone healing but did impact the immune response. The RTC induced upregulation of $CD4^+$ T cells was consistent with collagen's known bioactivity and potential antigenicity and highlights the cytocompatibility of PEG-GEL. Current work is discriminating the T helper and regulatory subtypes and $NO^{\cdot}$'s modulation of TGF-β1 signaling.

SIGNIFICANCE/CLINICAL RELEVANCE: A synergistic interaction between nitric oxide and TGF-β1 was found to promote osteogenesis in segmental defects at low cytokine concentrations. This is the basis for a novel therapeutic that enables early treatment of contaminated open bone wounds in low resource settings such as rural communities and low-income countries.