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Minocycline enhances the mesenchymal stromal/stem cell pro-healing phenotype in triple antimicrobial-loaded hydrogels

Alberto Daniel Guerra^a, Warren E. Rose^a, Peiman Hematti^b, and W. John Kao^{a,c,d,*,1}

^aSchool of Pharmacy, Division of Pharmaceutical Sciences, Pharmacy Practice Division, University of Wisconsin-Madison, 777 Highland Avenue, Madison, WI 53705, USA

^bSchool of Medicine and Public Health, Department of Medicine, Wisconsin Carbone Cancer Center, University of Wisconsin-Madison, 1685 Highland Avenue, Madison, WI 53705, USA

^cCollege of Engineering, Department of Biomedical Engineering, University of Wisconsin-Madison, 1415 Engineering Drive, Madison, WI 53706, USA

^dSchool of Medicine and Public Health, Department of Surgery, University of Wisconsin-Madison, 1685 Highland Avenue, Madison, WI 53705, USA

Abstract

Mesenchymal stromal/stem cells (MSCs) have demonstrated pro-healing properties including an anti-inflammatory cytokine profile and the promotion of angiogenesis via expression of growth factors in pre-clinical models. MSCs encapsulated in poly(ethylene glycol) diacrylate (PEGdA) and thiolated gelatin poly(ethylene glycol) (Gel-PEG-Cys) crosslinked hydrogels have led to controlled cellular presentation at wound sites with favorable wound healing outcomes. However, the therapeutic potential of MSC-loaded hydrogels may be limited by non-specific protein adsorption on the delivery matrix that could facilitate the initial adhesion of microorganisms and subsequent virulent biofilm formation. Antimicrobials loaded concurrently in the hydrogels with MSCs could reduce microbial bioburden and promote healing, but the antimicrobial effect on the MSC wound healing capacity and the antibacterial efficacy of the hydrogels is unknown. We demonstrate that minocycline specifically induces a favorable change in MSC migration capacity, proliferation, gene expression, extracellular matrix (ECM) attachment, and adhesion molecule and growth factor release with subsequent increased angiogenesis. We then demonstrate that hydrogels loaded with MSCs, minocycline, vancomycin, and linezolid can significantly decrease bacterial bioburden. Our study suggests that minocycline can serve as a dual mechanism for the regenerative capacity of MSCs and the reduction of bioburden in triple antimicrobial-loaded hydrogels.

Keywords

Mesenchymal stromal/stem cells; Hydrogel; Minocycline; *Staphylococcus aureus*; Wound healing

*Corresponding author at: 777 Highland Avenue, 7123 Rennebohm Hall, Madison, WI 53705, USA.

¹Current address: 10/F Knowles Building, Pokfulam, Hong Kong.

Disclosures

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1. Introduction

Bone marrow-derived mesenchymal stromal/stem cells (MSCs) have demonstrated pro-healing properties through multiple mechanisms: an anti-inflammatory cytokine expression profile, decreased fibrotic granular tissue, enhanced keratinocyte proliferation, inhibited myofibroblast differentiation, and elevated proliferation of microvascular endothelial cells with subsequent angiogenesis [1–8]. The wound healing properties of MSCs are maintained when MSCs are encapsulated in a poly(ethylene glycol)-diacrylate (PEGdA) and thiolated gelatin poly(ethylene glycol) (Gel-PEG-Cys) crosslinked hydrogel utilized for spatially and temporally controlled cellular presentation and the promotion of full-thickness cutaneous wound healing [9–12]. The hydrogel utilizes a modified gelatin with cysteine through a PEG linker that leads to cytoplasmic spreading and the formation of cellular networks that improves cellular delivery and extends survival time of MSCs with increased residence time at the wound site compared to an intravenous injection [9,10,12]. However, the therapeutic potential of biomaterial cell-based therapies may be limited by non-specific protein adsorption on the delivery matrix similar to that of implants. This in turn could facilitate the initial adhesion and replication of wound-associated microorganisms such as *Staphylococcus aureus* (SA), and a quorum sensing response to high cell density that leads to SA attachment, virulence expression and production, and eventual biofilm formation [13–17]. SA within biofilms produce extracellular polymer substance that results in bacterial resistance to immune cell recognition and elimination as well as reduced antibacterial activity [14]. Biofilm bacteria remain virulent by creating an acidic and hypoxic microenvironment that contributes to primary immune cell death and tissue necrosis. Sloughing of bacteria from the implant surface results in a dysfunctional innate immune response through high secretion of proteases, reactive oxygen species, and pro-inflammatory cytokines at the implant site [18,19]. Virulent SA that form biofilms is one of the most common pathogens in non-healing wounds. Over half of patient chronic wound samples have shown the presence of biofilms, and SA has been used in infected rat wound models [20–22]. Opportunely, the hydrogel system can be loaded with multiple prophylactic antimicrobials with controlled release kinetics [23–25]. We have previously demonstrated that hydrogels loaded concurrently with MSCs and minocycline significantly lowers SA colony forming potential in solution while maintaining MSC viability and multipotency [26]. However, at long culture times (16 h), planktonic SA growth was observed that could lead to enhanced bioburden [18,19,26,27]. The addition of other broad-spectrum antimicrobials to the hydrogel system to prevent bacterial growth through multiple mechanisms may lead to increased antimicrobial efficacy. However, the pharmacological effects of antimicrobials on the MSC wound healing profile and the bacterial growth inhibition of MSC and triple antimicrobial-loaded hydrogels has not been investigated. In this study, we develop hydrogels loaded with MSCs and an antimicrobial combination that includes minocycline, vancomycin, and linezolid and investigate the mechanistic insights of the pro-healing outcome by quantifying the antimicrobial-induced effects on MSC migration capacity, proliferation, gene expression, extracellular matrix (ECM) attachment, cytokine, adhesion molecule, and growth factor production with subsequent angiogenesis potential. We then determine the release kinetics of the antimicrobials from the MSC-loaded hydrogels

and then determine the antibacterial capacity of MSC and triple antibiotic-loaded hydrogels against SA.

2. Materials and methods

2.1. Cell isolation and culture

2.1.1. MSC isolation, characterization and culture—MSCs were isolated from discarded filters of bone marrow harvests of healthy adult human donors based on a protocol approved by the University of Wisconsin Hospital and Clinics Regulatory Committee per our published protocols [10,28]. Isolated MSCs at passage 4 were characterized for positive and negative markers via flow cytometry, and for multidifferentiation potential as previously described [10,28,29]. MSCs were cultured in 75 cm² tissue culture flasks (TPP, St. Louis, MO) with Dulbecco's Modified Eagle Medium (DMEM, Cellgro Mediatech, Inc., Corning, NY), 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 2 mM non-essential amino acids (NEAA) with medium changes every 3–5 days. Only MSCs passage 4–8 were used in this study.

2.1.2. S. aureus culture—SA (ATCC strain #29213, Manassas, VA) culture vials stored at –80 °C were thawed, plated on trypticase soy agar (TSA, Becton Dickinson, Sparks, MD) Petri dishes (Fischer Scientific) and cultured as previously described [26]. The SA culture was allowed to proliferate in suspension on a shaker at 170 rpm at 37 °C until desired growth phase optical density was achieved (OD₆₀₀ = 0.1, 1 × 10⁴ CFU/mL) as measured by the spectrophotometer (Nanodrop 2000x UV–vis Spectrophotometer, Thermo Scientific). SA at OD₆₀₀ = 0.1 represents the pre-log growth phase (lag phase) in which bacteria will have to replicate at the wound site to establish a clinical infection, representing a wound infection model for most clinical cases [30,31].

2.1.3. HUVEC culture—Human umbilical vein endothelial cells (HUVECs) (ThermoFisher Scientific) were thawed and cultured in 75 cm² tissue culture flasks (TPP) with medium 200 (ThermoFisher Scientific) with 10% low serum growth supplement (ThermoFisher Scientific) with medium changes every 3–5 days. Only passage 4–8 HUVECs were used in this study.

2.2. Development of MSC and antimicrobial-loaded hydrogels

2.2.1. Toxicity of MSCs Exposed to drug cocktails—MSCs were plated at 40,000 cells/mL in 96-well TCPS plates (BD Biosciences). A 5 mg/mL stock of minocycline (Research Products International, Mt. Prospect, IL), vancomycin (Dot Scientific Inc., Burton, MI), and linezolid (AK Scientific Inc., Union City, CA) were made in MSC growth medium and were diluted to appropriate working solutions. The appropriate amount of each working solution was added to make the following drug conditions of minocycline + vancomycin + linezolid in µg/mL: 25/50/5, 35/30/7.5, 50/40/10, 75/60/15, and 100/80/20, respectively, determined from independent preliminary MSC toxicity studies of each antimicrobial. Plates were incubated for 1, 3, 5, and 7 days. Respective media was changed after 3 days in the 5 and 7 day treatment groups to avoid cell death as a result of a lack of nutrients and a buildup of toxic metabolism by-products in the medium. After respective

treatment times, the medium was removed from the well and a LIVE/DEAD® (Invitrogen) stain was applied with subsequent imaging and fluorescence quantification followed by a CellTiter-Blue® (Promega, Madison, WI) stain with subsequent quantification as previously described [26].

2.2.2. Hydrogel preparation—Hydrogel formulations were made in PBS to make a 0.5% (w/v) Irgacure 2959 photoinitiator (BASF, Ludwigshafen, Germany), 10% (w/v) PEGdA and 10% (w/v) Gel-PEG-Cys sterile-filtered solution prepared and characterized as described previously [32,33]. A MSC suspension was added into the formulation at 1×10^6 cells/mL. For hydrogel samples loaded with antimicrobials, 1 mg/mL stocks were made in PBS and the appropriate amount was added to a solution containing photoinitiator, PEGdA, and Gel-PEG-Cys in PBS. The antimicrobial load formulation for preliminary MSC screening was selected based on the toxicity screening stated above. Hydrogels loaded concurrently with MSC and antimicrobials individually or in combination were prepared following similar procedures as described above. The formulations were then pipetted (100 μ L) into a 10 mm glass-bottom Petri dish (In Vitro Scientific, Sunnyvale, CA) with a circular recess and then polymerized by exposing to UV light ($\lambda_{\text{max}} = 365$ nm, 100 W/cm²) for 2 min. MSC medium (2 mL) with corresponding drug concentrations (when applicable) was added to the tissue culture Petri dish to ensure the concentration of antimicrobials in the hydrogels was maintained through the swelling process, and the hydrogels were swollen for 24 h at 37 °C.

2.2.3. MSC Characterization in prepared hydrogels—After 24 h of swelling, hydrogels encapsulated with MSCs (with and without drug combinations) were carefully transferred to 24 well tissue culture plates (Celltreat®, Shirley, MA) and treated with LIVE/DEAD® and imaged on an epifluorescent scope followed by a CellTiter-Blue® dilution as stated above, but with a 16 hour incubation to allow the dye to reach the cells through the hydrogel network for metabolic reduction [26]. Live and dead MSC cell counts were conducted using ImageJ software (National Institutes of Health, MD).

MSCs were encapsulated in the hydrogel and swollen with and without drug combinations as stated above. After 24 h of swelling, the hydrogels were analyzed for differentiation potential by transferring them to a 24 well tissue culture plate (Celltreat®) with either osteo NHdiff medium (10 days), adipo NHdiff medium (14 days), chondo NHdiff medium (14 days), or MSC expansion media (14 days) with medium changes every 4 days. After respective differentiation times, the final cell medium supernatants were collected and osteocyte, adipocyte, and chondrocyte differentiation was determined by assaying established biomarkers using a human osteocalcin ELISA (Invitrogen), a human adiponectin ELISA kit (Invitrogen), and a human aggrecan ELISA (Invitrogen), respectively, following the manufacturer's instructions [34–36].

2.3. Antimicrobial induction of MSC pro-healing functions

2.3.1. Cell migration—MSC cell migration was assessed using a CytoSelect™ 24-Well Wound Healing Assay (Cell Biolabs, Inc.). Briefly, MSCs were plated at 100,000 cells/well in TCPS wells with MSC media alone or MSC media containing 50 μ g/mL minocycline, 40

µg/mL vancomycin, 10 µg/mL linezolid, or 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid incubated with the provided inserts at 37 °C overnight. Inserts were removed and plates were incubated at 37 °C for 24 h as suggested by the manufacturer. The media was then aspirated and wells were imaged (Nikon Eclipse TE300) and gap closure was quantified using ImageJ software using the area of the insert as the original area of the gap: $[(\text{area of original gap} - \text{remaining area of gap}) / \text{area of original gap}] \times 100\%$.

2.3.2. Proliferation assay—MSC proliferation induction from antimicrobials was assessed using a CellTrace™ CFSE Cell Proliferation Kit (ThermoFisher Scientific). Briefly, MSCs (50,000 cells/well) were stained with 1 µM of CellTrace™ stain and plated on 24 well TCPS plates (Celltreat®) with MSC media alone or MSC media containing 50 µg/mL minocycline, 40 µg/mL vancomycin, 10 µg/mL linezolid, or 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid. After 1 and 3 days of culture, MSCs were harvested via trypsin and resuspended in a 10% FBS in PBS (FACS buffer) solution and analyzed on a BD Accuri C6 Flow Cytometer (BD Biosciences) to determine MSC proliferation rates. Proliferation index of each sample was determined using proliferation analysis via FlowJo software (Tree Star).

2.3.3. MSC adhesion assay—MSC adhesion to ECM proteins was analyzed using a 48-well ECM Array Cell Adhesion Assay (Cell Biolabs, Inc.). Briefly, MSCs were plated at 100,000 cells/well in ECM protein-coated wells and cultured for 90 min with MSC media alone or MSC media containing 50 µg/mL minocycline, 40 µg/mL vancomycin, 10 µg/mL linezolid, or 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid and analyzed for attachment as suggested by the manufacturer.

2.4. MSC gene expression and soluble factor release

MSCs in culture were plated in 24 well TCPS plates (CellStar®) at 100,000 cells/well and MSCs in hydrogels were loaded at 1×10^6 - cells/mL (100,000 cells per hydrogel). MSCs were cultured or encapsulated and swelled with the following media conditions: MSC media alone, MSC media containing 50 µg/mL minocycline, 40 µg/mL vancomycin, 10 µg/mL linezolid, or 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid; with all conditions being as stated or in combination with 20 ng/mL tumor necrosis factor alpha (TNF-α, Fisher Scientific) + 20 ng/mL interferon gamma (IFN-γ, Fisher Scientific) [37,38]. Hydrogels treated with 20 ng/mL TNF-α + 20 ng/mL IFN-γ only had the inflammation factors present in the swelling media and not in the encapsulated hydrogels as the antimicrobials were. After 48 h of culture or hydrogel swelling, supernatants were removed for subsequent soluble factor release using ELISA kits with corresponding assay antibodies and standards: interleukin 6 (IL-6, R&D Systems, Inc., Minneapolis, MN), interleukin 10 (IL-10, R&D Systems, Inc.), intercellular adhesion molecule 1 (ICAM-1, R&D Systems, Inc.), vascular endothelial growth factor (VEGF, R&D Systems, Inc.), fibroblast growth factor (FGF, R&D Systems, Inc.), and hepatocyte growth factor (HGF, Cell Applications, Inc., San Diego, CA). MSC RNA was isolated first by lysing MSCs in culture using 0.5 mL of TRIzol reagent (ThermoFisher Scientific, Waltham, MA) per well in culture. MSCs in hydrogels were placed in liquid nitrogen in a granite dish and crushed with a pestle until the hydrogel was in a yellow powder followed by the addition of 0.5 mL of TRIzol reagent and

transfer to an Eppendorf tube [39]. The remaining RNA extraction protocol remains the same for both cultured MSCs and MSCs in hydrogels. 100 μ L of 1-bromo-3-chloropropane (BCP, Sigma Aldrich) was added to each sample, vortexed for 15 s, incubated at room temperature for 3 min, and centrifuged for 15 min at 12,000g at 4 °C. The top clear layer was transferred to a new tube and 1 volume of 70% ethanol was added and mixed by pipetting. The solution was then transferred to a spin column from an RNeasy Mini Kit (Qiagen, Valencia, CA) and RNA was isolated by continuing the kit protocol. Isolated total RNA concentration was measured using a Nanodrop 2000x UV-vis Spectrophotometer (Thermo Scientific) and reverse transcription was performed with 5 μ g of total RNA using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, California) in an iCycler thermocycler (Bio-Rad). RT-PCR was performed on synthesized cDNA using a StepOnePlus Real-Time PCR System (ThermoFisher Scientific) using TaqMan Expression Assay Probes for glucose-6-phosphatase isomerase (*GPI*), vascular cell adhesion molecule 1 (*VCAM1*), galectin 1 (*LGALS1*), TEK tyrosine kinase (*TEK*), colony stimulating factor 1 (*CSF1*), and signal transducer and activator of transcription 2 (*STAT2*, Life Technologies, Carlsbad, CA). Data pertaining to mRNAs were collected quantitatively and the CT number was corrected by CT readings of corresponding internal control of *GPI* [40].

2.5. Endothelial tube formation assay

MSCs were encapsulated and swelled in hydrogels loaded at 1×10^6 cells/mL (100,000 cells per hydrogel) for 48 h at with corresponding antimicrobial concentrations as stated above. After 48 h of MSC-loaded hydrogel swelling, HUVECs were stained with 2 μ M calcein (Invitrogen) in PBS for 30 min at 37 °C in the tissue culture flasks followed by a PBS wash. Tube formation of HUVECs was detected in media extracted from hydrogel swelling using a Cultrex In Vitro Angiogenesis Assay (R&D Systems) and HUVEC tube formation was analyzed via imaging each well at 4 separate points using an epifluorescent microscope and quantified by counting the number of branch points on each image [41].

2.6. In vitro drug release kinetics

Hydrogels were formulated and polymerized as stated above using two formulations: 50 μ g/mL minocycline + 40 μ g/mL vancomycin + 10 μ g/mL linezolid with MSCs at 1×10^6 cells/mL and hydrogels with no antimicrobials but with MSCs at 1×10^6 cells/mL. Hydrogels were carefully removed from 10 mm glass-bottom Petri dishes after polymerization and placed in a scintillation vial with 250 μ L of PBS and incubated at 37 °C. At 0.25, 1, 2, 4, 6, 12, 24, 48, and 72 h, 2 μ L of PBS was removed from the scintillation vials at the absorbance was measured at 350 nm, 200 nm, and 250 nm for the presence of minocycline, vancomycin, and linezolid, respectfully, and compared to values of hydrogel loaded with MSCs only on a Nanodrop 2000x UV-vis Spectrophotometer [42–44].

2.7. Hydrogel + SA co-culture and characterization

Hydrogels were made as described above using optimized antimicrobial dosages and encapsulated with: (a) no encapsulated cells or solutes (b) MSCs (1×10^6 cell/mL) or (c) MSCs (1×10^6 cell/mL) + 50 μ g/mL minocycline + 40 μ g/mL vancomycin + 10 μ g/mL linezolid. Planktonic SA at the pre-log growth phase ($OD_{600} = 0.1$, 1×10^4 CFU/mL) was prepared as stated above and centrifuged at 5000g for 10 min and resuspended in the same

volume of MSC growth medium [45]. Hydrogels were then transferred to 24 well tissue culture plates (CellStar®) with 800 µL of the planktonic SA in MSC growth medium suspension and incubated at 37 °C as previously described [26]. The same amount of SA in MSC growth medium and in TSB was added to the plate as positive controls. The amount of SA solution added to each well was determined by the amount needed to cover the entire volume of the hydrogel in the well. At 2, 4, and 18 h, 50 µL of the SA solution was removed and serially diluted (1:10) for colony forming ability enumeration as previously described [26]. After 18 h of co-culture, hydrogels were then transferred to another 24-well plate (Celltreat®) for LIVE/DEAD® analysis as described above followed by a 1:10 CellTiter-Blue® analysis with a 2 h incubation at 37 °C to measure the metabolic activity of SA attached to the hydrogel surface. Lastly, SA cell attachment to the hydrogel surface was done using a gentian violet stain as previously described [15].

2.8. Statistical methods

All assays were conducted with four replicates per condition ($n = 4$). A two-way ANOVA for multiple comparisons was used with a Bonferroni test assessment to determine p values for all assays with 3 or more conditions. An unpaired two-tailed Student's t test was used to determine p values for all assays with only 2 conditions. A probability value $P < 0.05$ was considered statistically significant. Error bars represent the standard deviation of the specific group mean.

3. Results

3.1. Antimicrobial-induced MSC toxicity

To determine hydrogel-loading concentrations of antimicrobial combinations that maintain MSC metabolic viability, exogenous dosing of MSCs with respective drug combinations was first performed. Following 7 days of MSC culture in the presence of minocycline + vancomycin + linezolid, MSCs demonstrated no significant decrease in viability, metabolic activity, or growth density when cultured with 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid (Fig. 1A–B).

Live cell percentage, metabolic activity, and differentiation potential of MSCs in PEGdA + Gel-PEG-Cys hydrogels with drug combinations was then quantified as a preliminary indication of maintained functionality. After 24 h of swelling, encapsulated MSCs demonstrated no significant differences in live cell percentages or in metabolic activity in the presence of the concurrently loaded drug formulation compared to MSCs in hydrogels with no antimicrobials (Fig. 1C). MSCs loaded in hydrogels loaded concurrently with minocycline + vancomycin + linezolid at the concentrations stated above demonstrated multipotent differentiation potential onto osteocytes, adipocytes and chondrocytes as comparable to MSCs loaded in hydrogels without drugs. The secretion of osteocalcin, adiponectin and aggrecan were utilized as trilineage differentiation phenotype markers, respectively. Moreover, MSCs in hydrogels loaded with minocycline + vancomycin + linezolid secreted significantly higher adiponectin than MSCs in hydrogels not loaded with antimicrobials (Fig. 1D).

3.2. Antimicrobial-induced MSC pro-healing functions

MSCs were analyzed for wound healing functionality with antimicrobials to determine if the antimicrobials had any effect on the MSC wound healing phenotype. MSCs cultured with 50 µg/mL minocycline alone and with 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid demonstrated significantly higher migration into the gap after 24 h of culture compared to MSCs cultured without antimicrobials (Fig. 2A–B). MSCs cultured with 50 µg/mL minocycline alone and with 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid demonstrated a significantly higher proliferation index after 3 days of culture compared to MSCs cultured with no antimicrobials, although there was no difference in proliferation index after 1 day of culture between treatment conditions (Fig. 2C). MSCs cultured with or encapsulated and swollen in hydrogels with 50 µg/mL minocycline or 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid expressed significantly higher levels of *LGALS1* and *TEK* after 48 h of culture or hydrogel swelling compared to MSCs cultured or swollen in hydrogels with no antimicrobials. There were no differences in the expression of *VCAMI* between any MSC culture or hydrogel conditions (Fig. 2D–E).

MSCs cultured with 40 µg/mL vancomycin demonstrated significantly higher cell attachment to fibronectin, collagen I, and fibrinogen after 90 min of culture compared to MSCs cultured with no antimicrobials (Fig. 3). MSCs cultured with 50 µg/mL minocycline demonstrated significantly higher attachment to fibronectin, collagen I, collagen IV, laminin I, and fibrinogen compared to MSCs cultured with 40 µg/mL vancomycin and compared to MSCs cultured with no antimicrobials. MSCs cultured with 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid demonstrated significantly higher attachment to fibronectin, collagen I, collagen IV, laminin I, and fibrinogen compared to MSCs cultured with 40 µg/mL vancomycin and compared to MSCs cultured with no antimicrobials; and demonstrated significantly higher attachment to fibronectin, collagen I, and collagen IV compared to MSCs cultured with 50 µg/mL minocycline (Fig. 3).

3.3. MSC immunomodulation potential in response to antimicrobials

MSCs in culture and encapsulated in hydrogels secreted significantly higher levels of IL-6 when stimulated with 20 ng/mL TNF-α + 20 ng/mL IFN-γ between all respective antimicrobial conditions. There was no difference in IL-6 secretion between antimicrobial-only conditions and between antimicrobial conditions with 20 ng/mL TNF-α + 20 ng/mL IFN-γ in culture or in hydrogels. There was no difference in MSC IL-10 secretion in culture or in hydrogels between conditions with antimicrobials-only and antimicrobials with 20 ng/mL of TNF-α + 20 ng/mL IFN-γ, as well as no difference between any antimicrobial-only conditions or any antimicrobial conditions with 20 ng/mL of TNF-α + 20 ng/mL IFN-γ. MSCs in culture and encapsulated in hydrogels secreted significantly higher levels of ICAM-1 when stimulated with 20 ng/mL TNF-α + 20 ng/mL IFN-γ between all respective antimicrobial conditions in culture and in hydrogels. There was no difference in ICAM-1 secretion between antimicrobial-only conditions in culture and in hydrogels. MSCs cultured or encapsulated and swelled in hydrogels with 20 ng/mL of TNF-α + 20 ng/mL IFN-γ in combination with 50 µg/mL minocycline or in combination with 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid secreted significantly higher levels of

ICAM-1 compared to MSCs cultured or encapsulated and swelled in hydrogels with media only containing 20 ng/mL of TNF- α + 20 ng/mL IFN- γ (Fig. 4A–B).

MSCs demonstrated a significant increase in expression of *CSF1* and *STAT2* when cultured or encapsulated and swollen in hydrogels with medium containing 20 ng/mL of TNF- α + 20 ng/mL IFN- γ compared to the same antimicrobial-containing media condition with no inflammatory factors. There was no difference in *CSF1* or *STAT2* expression between antimicrobial-only conditions and between antimicrobial conditions with 20 ng/mL of TNF- α + 20 ng/mL IFN- γ in culture or in hydrogels (Fig. 4C–D).

3.4. MSC growth factor secretion in response to antimicrobials and subsequent endothelial tube formation

Growth factor secretion and subsequent angiogenesis potential were analyzed to determine if there was an antibiotic-induced change in the secretion of growth factors from MSCs that may increase angiogenesis in healing wound beds. MSCs cultured or encapsulated and swollen in hydrogels containing 50 μ g/mL minocycline or 50 μ g/mL minocycline + 40 μ g/mL vancomycin + 10 linezolid μ g/mL secreted significantly higher levels of VEGF. There was no difference in secretion of FGF or HGF between culture or hydrogel conditions (Fig. 5A–B). HUVECs cultured with media conditioned from 48 h of swelling of MSC-loaded hydrogels with 50 μ g/mL minocycline or 50 μ g/mL minocycline + 40 μ g/mL vancomycin + 10 μ g/mL linezolid demonstrated a significant increase in tube formation branch points after 12 h of culture compared to HUVECs cultured with media conditioned from 48 h of swelling of MSC-loaded hydrogels with no antimicrobials (Fig. 5C–D). HUVECs cultured with MSC media with 10 μ M sulforaphane was utilized as a negative control and HUVECs cultured with fresh MSC media with 50 μ g/mL minocycline was used to show that minocycline did not have a direct effect on HUVEC tube formation.

3.5. In vitro antimicrobial release from MSC-loaded hydrogels

Hydrogels loaded with minocycline + vancomycin + linezolid with MSCs demonstrated a burst release of all three antimicrobials over a 6 h period. About 50% of the loaded dose of minocycline and vancomycin was released, corresponding to 2.5 μ g and 2 μ g, respectively. About 90% of the loaded dose of linezolid was released, corresponding to 0.9 μ g (Fig. 6).

3.6. In vitro SA growth and attachment inhibition by hydrogels loaded with MSCs and antimicrobials

Analysis of SA growth, SA attachment to the hydrogels, SA metabolic activity, and maintenance of MSC viability was determined by co-culturing hydrogel formulations in a solution of planktonic SA at the early exponential phase ($OD_{600} = 0.1$). After 2, 4 and 18 h of co-culture, SA colony forming ability was significantly lowered in the presence of hydrogels loaded with MSCs + minocycline + vancomycin + linezolid (Fig. 7A). After 18 h of co-culture, planktonic SA attachment to the hydrogel surface was significantly lower, metabolic activity of SA on the hydrogel was significantly lower, and MSC live cell percentage was significantly higher when hydrogels were loaded with MSCs + minocycline + vancomycin + linezolid (Fig. 7B–D).

4. Discussion

Our previous study with MSC and minocycline-loaded hydrogels demonstrated an *in vitro* reduction of SA growth, but with a resurgence of growth after 16 h of co-culture [26]. In this study, we loaded the hydrogels with a combination of minocycline, vancomycin, and linezolid to prevent SA growth through prokaryotic protein synthesis inhibition and cell wall production inhibition in an effort to prevent a resurgence of SA growth at long culture times and reduce bacterial bioburden [46–49]. The effect of the loaded antimicrobials on the MSC wound healing capacity is unknown and was investigated in this study. The induction of cell migration and proliferation are critical for MSCs to localize to the open wound site and have a strong cytokine and growth factor paracrine effect [50]. Our results suggest that minocycline could increase the strength of the MSC wound healing response through localization and through higher amounts of cells through increased proliferation. *VCAM1* is an adhesion molecule known to mediate cell migration, however, it was not upregulated in the presence of minocycline [51]. *LGALS1* modulates cell-cell and cell-matrix interactions and may stimulate angiogenesis when expressed by MSCs [52]. The upregulation of *LGALS1* in the presence of minocycline may have contributed to increased ECM attachment that could facilitate migration, and may have contributed to increased angiogenesis seen in HUVECs cultured with media conditioned from MSCs in hydrogels treated with minocycline. *TEK* is a tyrosine kinase receptor that can lead to proliferation through growth factor binding and is known to induce angiogenesis in endothelial cells when bound to growth factors [53]. The increased MSC VEGF secretion induced by minocycline may have contributed an autocrine effect on the increased *TEK* expressed receptors that could lead to increased proliferation of MSCs after culture with minocycline, as the receptor responds to growth factor binding to induce downstream signaling that can lead to proliferation [53]. Due to the minocycline-induced increased expression of *LGALS1*, we investigated the attachment of MSCs to ECM proteins in the presence of antibiotics. MSCs on collagen membranes have shown to induce accelerated wound healing [54]. MSCs on ECM-derived scaffolds can increase the longevity and functionality of wound healing, and MSCs on these ECM scaffolds maintain integrity and vitality for up to 14 days [55]. Fibronectin and collagen I can induce MSC chemotaxis and haptotaxis [56]. MSC attachment to fibrinogen has been shown to upregulate immunosuppression by inducing an increased secretion of IL-6 and indoleamine dioxygenase (IDO) while mitigating T cell proliferation [57]. MSC attachment to laminin controls proliferation and is observed in a myogenic differentiation study [58]. The synergistic effect of vancomycin and minocycline inducing increased attachment to the ECM proteins may increase MSC viability and wound healing functionality at the wound site that could potentially contribute to accelerated wound healing.

Inflammation accompanies tissue injury for the clearance of damaged tissues and invaded pathogens, and these environments are rich in the pro-inflammatory cytokines TNF- α and IFN- γ ; however, excessive activation of immune cells may hinder repair and MSCs respond to these inflammatory cytokines, eliciting an immunosuppressive response that can reduce inflammation and create a favorable wound healing environment [59,60]. MSC immunosuppression abilities were not hindered by the addition of the antimicrobials. IL-6 is

a cytokine that is typically considered as pro-inflammatory but in certain conditions it could act as an anti-inflammatory cytokine and can promote angiogenesis as well, and IL-10 is an anti-inflammatory cytokine that contributes to the downregulation of T cell proliferation [37,60,61]. The increase in IL-6 and the lack of increase in IL-10 secretion by MSCs due to inflammation has been observed previously, and the antimicrobials had no effect on MCS IL-6 and IL-10 secretion [62]. ICAM-1 is a critical MSC immunosuppressive molecule that is produced by MSCs in the obligatory presence of IFN- γ , and produced in larger amounts from a synergistic effect with TNF- α [63]. ICAM-1 secretion by MSCs leads to increased adhesion between MSCs and T cells where MSCs can produce NO to inhibit T cell proliferation and other immune responses [59]. MSC immunosuppression is significantly reversed when ICAM-1 is genetically deleted or functionally blocked, so it was imperative that we demonstrated ICAM-1 secretion from MSCs when stimulated with IFN- γ and TNF- α in the presence of antimicrobials [63]. The synergistic effect of increased ICAM-1 secretion from minocycline with inflammatory cytokines in addition to maintained IL-6 and IL-10 secretion suggests that minocycline may induce a stronger immunosuppressive paracrine effect in MSCs that could potentially reduce inflammation in wounds.

CSF1 upregulation leads to the proliferation of macrophages, and macrophage subpopulations may reduce inflammation and enhance angiogenic capacities when proliferating at high rates [64,65]. *STAT2* is an immunosuppressive gene upregulated in the presence of IFN- γ and TNF- α that acts a transcription factor to induce the expression of more immunosuppressive genes that modulate wound healing [66,67]. Antimicrobials having no effect on *CSF1* and *STAT2* upregulation suggest that induced macrophage subpopulation proliferation and the *STAT2* immunomodulatory pathway are not enhanced or attenuated in the antimicrobial-loaded hydrogels.

Many wounds have an inadequate microvasculature, which is required for oxygen and nutrient supply as well as for the removal of metabolic waste products and the migration of immune and progenitor cells to the wound site [68]. MSCs can induce angiogenesis through the secretion of growth factors to promote the proliferation and subsequent tube formation of microvascular endothelial cells which ultimately establishes a long-lasting functional vascular network [4–8]. MSCs can secrete VEGF, FGF, and HGF and this paracrine effect can induce angiogenesis, increase endothelial permeability for the flow of small molecules and lymphocytes to the wound site, prevent apoptosis, stimulate autocrine-induced MSC proliferation, and inhibit epithelial to myofibroblast transition [69–74]. These growth factors and the cytokines discussed previously being present in the hydrogel swelling supernatant confirms that MSC-secreted growth factors and cytokines can penetrate the hydrogel network and reach the wound site to enhance wound healing. The increase in tube formation in HUVECs treated with media conditioned from MSCs in hydrogels loaded and swelled with minocycline may be due to the minocycline-induced increased MSC secretion of VEGF, and suggests the potential for minocycline-enhanced MSCs to generate an increased microvasculature network to increase oxygen, nutrient, and lymphocyte delivery at the wound site [6–8]. The antimicrobials did not attenuate the secretion of FGF or HGF in MSCs, suggesting the effect of these growth factors are maintained in the antimicrobial-loaded hydrogels.

Release of the antimicrobials from the hydrogels is another necessary component of the hydrogel system to prevent of SA growth, attachment, and metabolic activity on the hydrogel surface and in the hydrogel environment as SA has shown to cause infection in acute and chronic cutaneous wounds, has shown to create biofilms in wounds and on biomaterial implants, and has been shown to cause a rapid progression of infection and biofilm formation [13,18,20]. All three antimicrobials released within 6 h corresponds to the delivery of amounts surpassing the minimum inhibitory concentrations for SA that would be delivered with spatial control to the wound environment as compared to systemic antibiotic administration [75–77]. SA can secrete leukocidins, haemolysins, exotoxins, and enterotoxins in addition to the formation of an anoxic and acidic environment created by metabolic byproducts of SA that have converted to a gene expression profile favoring biofilm development, which can lead to device failure, chronic wound formation, and increased patient morbidity [20,78–81]. Soluble factors released from the biofilm can negatively impact the cellular functions of MSCs, as biofilm phenotype SA have shown to reduce the viability of MSCs via the activation of apoptosis by increasing caspase activity in MSCs, decreasing MSC multilineage potential, and inhibiting MSC migration to wound site [82]. Although MSCs have shown to release the antimicrobial peptide LL-37, our previous study has shown that MSCs alone cannot inhibit colony-forming abilities of SA and that MSC and minocycline-loaded hydrogels can only inhibit colony-forming abilities of SA for less than 16 h [26,83]. There was a clear need to incorporate multiple antimicrobials into the hydrogel to prevent bacterial bioburden and preserve the MSC wound healing potential. In this study, we demonstrate that minocycline can serve as a dual mechanism for the regenerative capacity of MSCs and the reduction of bioburden in triple antimicrobial-loaded hydrogels.

5. Conclusions

Minocycline induces a favorable change in MSC migration capacity, proliferation, ECM attachment, and adhesion molecule release. Minocycline within PEGdA and Gel-PEG-Cys composite hydrogels induces a favorable change in gene expression and growth factor release, which can penetrate through the hydrogel network and induce angiogenesis. Triple antimicrobial-loaded hydrogels reduce bacterial bioburden and preserve MSC viability in the presence of bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2017.01.021>.

Statement of Significance

Wound healing is a complex biological process that can be hindered by bacterial infection, excessive inflammation, and inadequate microvasculature. In this study, we develop a new formulation of poly (ethylene glycol) diacrylate and thiolated gelatin poly(ethylene glycol) crosslinked hydrogels loaded with minocycline, vancomycin, linezolid, and mesenchymal stromal/stem cells that induces a favorable wound healing phenotype in mesenchymal stromal/stem cells and prevents bacterial bioburden on the hydrogel. This combinatorial approach to biomaterial development has the potential to impact wound healing for contaminated full thickness cutaneous wounds.

Fig. 1. MSC viability, metabolic activity, and functionality in combination with minocycline, vancomycin, and linezolid. (A) Cell viability (MFI 520 nm) of MSCs and metabolic activity (MFI 590 nm) of MSCs in culture with minocycline, vancomycin and linezolid. (B) Cell viability images (100× magnification, scale bar indicates 200 μm) of MSCs in culture with minocycline, vancomycin, and linezolid. (C) MSC live cell percentage and metabolic activity (MFI 590 nm) of MSCs in hydrogels (D) Differentiation potential of MSCs into osteocytes, adipocytes, and chondrocytes in hydrogels determined from the secretion of osteocalcin, adiponectin, and aggrecan respectively. *P < 0.05 versus 0/0/0 or no drug in differentiation media condition.

Fig. 2.

Antimicrobial-induced MSC cellular behavior. (A) Percent gap closure in the migration assay after 24 h culture. (B) Representative brightfield images (100× magnification, scale bar indicates 200 μm) of MSC gap closure after 24 h of culture. (C) MSC proliferation after 1 (■) and 3 (▒) days of culture. (D) Gene expression of cellular adhesion and tyrosine kinase genes in MSCs in response to antimicrobials in culture. (E) Gene expression of cellular adhesion and tyrosine kinase genes in MSCs in response to antimicrobials in hydrogels. * $P < 0.05$ versus no drug condition and versus 3 day no drug condition proliferation index.

Fig. 3.

MSC adhesion to extracellular matrix proteins with no antimicrobials (■), 50 µg/mL minocycline (▤), 40 µg/mL vancomycin (▒), 10 µg/mL linezolid (□), or 50 µg/mL minocycline + 40 µg/mL vancomycin + 10 µg/mL linezolid (▨) after 90 min of culture. $P < 0.05$ versus no drug condition (*), versus vancomycin condition (†), or versus minocycline condition (‡); all compared to specific ECM protein attachment.

Fig. 4.

MSC immunomodulation capacity in response to antimicrobials (■) and in response to antimicrobials with inflammation conditions, defined as stimulation with 20 ng/mL TNF- α + 20 ng/mL IFN- γ (■) after 48 h of culture or hydrogel swelling. (A) MSC immunomodulatory molecule secretion in culture. (B) MSC immunomodulatory molecule secretion in hydrogels. (C) MSC immunomodulatory gene expression in culture. (D) MSC immunomodulatory gene expression in in hydrogels. * $P < 0.05$ versus corresponding antimicrobial-only conditioned media. † $P < 0.05$ versus no drug condition with 20 ng/mL of TNF- α + 20 ng/mL IFN- γ only.

Fig. 5.

Growth factor release and resulting endothelial tube formation in response to media extracted from MSCs swollen in hydrogels for 48 h with antibiotics. (A) MSC growth factor release in culture. (B) MSC growth factor release in hydrogels. (C) Representative images of calcein-stained HUVECs after 12 h of culture on extracellular matrix extract gel (100x magnification, scale bar indicates 200 μ m). (D) Quantification of endothelial tube branch points. * $P < 0.05$ versus no drug condition.

Fig. 6.
In vitro release of minocycline, vancomycin, and linezolid from hydrogels loaded with all three antimicrobials and MSCs over 72 h.

Fig. 7.

In vitro antimicrobial efficacy of MSC and antimicrobial-loaded hydrogels. (A) Planktonic ($OD_{4600} = 0.1$) SA Log CFU/mL after 2 (■), 4 (□), and 18 (▒) hour co-culture with hydrogels. (B) Cell attachment (OD_{595}) of planktonic SA ($OD_{600} = 0.1$) to the hydrogel surface after 18 h co-culture of SA with hydrogels. (C) Metabolic activity (MFI 590) of planktonic SA ($OD_{600} = 0.1$) attached to the hydrogel surface after 18 h co-culture of SA with hydrogels. (D) Live cell percentage of MSCs in hydrogels after 18 h co-culture with SA. For SA Log CFU/mL enumeration, $P < 0.05$ versus No Hydrogel condition for 2 h (*), 4 h (†), 18 h (‡). For all other assays, * $P < 0.05$ versus no encapsulation condition and versus MSCs condition for % live MSCs.