Effect of co-administration of vancomycin and BMP-2 on co-cultured Staphylococcus aureus and W-20-17 mouse bone marrow stromal cells in vitro

A.H. Nguyen1, S. Kim1,2, W.J. Maloney2, J.C. Wenke3, Y. Yang1,2.

1 Department of Restorative Dentistry and Biomaterials, University of Texas Health Science Center at Houston, Houston, TX 77030
2 Department of Orthopedic Surgery, Stanford University, 94304
3 United States Army Institute of Surgical Research, 3698 Chambers Pass, Fort Sam Houston, Texas 78234

* Corresponding author:

Yunzhi Peter Yang, Ph.D.
Associate Professor
Department of Orthopedic Surgery
Stanford University
300 Pasteur Drive
Edwards R105
Stanford, CA 94305
Tel: 650-723-0772
Fax: 650-724-5401
Email: ypyang@stanford.edu
Abstract

In this study, we aim to establish an in vitro bacteria/bone cell co-culture model system and use this model for dose-dependence studies of dual administration of antibiotics and growth factors in vitro. We examined the effect of single or dual administration of the antibiotic vancomycin (VAN) at 0-16 μg/mL and bone morphogenetic protein-2 (BMP-2) at 0-100 ng/mL on both methicillin sensitive Staphylococcus aureus and mouse bone marrow stromal cells (W-20-17) in both mono and co-culture conditions. Cell metabolic activity, live/dead staining, dsDNA amount, and alkaline phosphatase activity were measured to assess cell viability, proliferation and differentiation. An interleukin-6 (IL-6) ELISA kit was used to test the bone cell inflammation response in the presence of bacteria. Our results suggest that when delivered together in co-culture, VAN and BMP-2 maintain their primary functions as an antibiotic and growth factor respectively. Most interestingly, this dual-delivery type of approach has shown itself to be effective at lower concentrations of VAN than an approach relying strictly on the antibiotic. It may be that BMP-2 enhances the cells’ proliferation and differentiation before becoming infected. In co-culture, a higher dosage of VAN may be necessary to effectively inhibit bacterial growth than that same treatment in monoculture of Staphylococcus aureus. This could mean that the co-culture environment may be limiting the efficacy of VAN, possibly by way of bacterial invasion of the bone cells. This co-culture study demonstrates a potential beneficial effect of the co-administration of antibiotics and growth factors when compared to treatment with antibiotic alone.
The regeneration of contaminated bony defects poses itself as one of the major areas of concern in the field of bone tissue engineering, especially with regard to extremity war traumas where infection of open type III fractures is all but certain [10, 34]. These bacterial infections complicate the already strenuous repair process, often resulting in slower union rates or even amputation [7, 23]. At the present, standard clinical care is a two-phase process. First, antibiotics are administered to stop any ongoing infection of the bony tissue if present. Second, a bone graft is implanted, and bone growth factors are delivered to facilitate the regrowth of natural bone tissue using the patient’s resident osteoblasts. While this treatment protocol is designed to prevent contamination and promote bone growth, recurrent infection following implantation of the graft continues to be a major barrier to generating consistently positive outcomes [2, 19, 30, 50].

One suggested approach to combating this issue is to use a specialized synthetic bone graft material capable of delivering both antibiotics and growth factors locally after implantation [9, 16, 26, 32, 48, 49, 52]. While antibiotic integration into bone grafts has been proven successful, less work has been done to deliver both an antibiotic and a growth factor together (either in series or parallel) [25, 26]. By limiting the need for the antibiotic to travel systemically, there will be higher antibiotic levels at the site of the wound and safe levels systemically, thus paving the way for resident osteoblasts to penetrate into the graft well before the offending bacteria are able to reach high confluence and form biofilms [39]. Moreover, the combined treatment with bone growth factors such as bone morphogenetic protein-2 (BMP-2) can accelerate the healing process in a simple, streamlined delivery approach [14, 20, 24, 29, 35, 38].

With the emergence of these complex dual-purpose grafts, the need for a flexible in vitro test
A system highly representative of *in vivo* conditions is of paramount importance in helping to smooth the natural transition into animal studies. Such a model system would allow for more realistic assessment of different clinical treatment options in a rapid, cost-efficient, and safe manner, especially with regard to testing possibly host-toxic therapies.

Here, we aim to establish an *in vitro* bacteria/bone cell co-culture model system and use this model for dose-dependence studies of the dual administration of antibiotics and growth factors *in vitro*. We examined the interactions between our two tested model cell lines (mouse bone marrow stromal cells W-20-17 and methicillin-sensitive *S. aureus* ATCC 6538) as well as their responses to various treatments with vancomycin (VAN) at 0-16 μg/mL and BMP-2 at 0 or 100 ng/mL both in mono and co-culture [13]. *S. aureus* is a highly infectious Gram-positive bacterium known for its ability to internalize itself within mammalian cells and is the most predominant cause of bone graft failures [2, 3, 8, 9, 11, 12, 15, 19, 30, 31, 33, 37, 46, 50]. W-20-17 is derived from mouse bone marrow stroma and has been used as an ASTM (F2131) standard test for *in vitro* biological activity of BMP-2. Previous studies showed that BMP-2 significantly stimulated alkaline phosphatase (ALP) activity in W-20-17 in a dose-dependent manner [27].

With so many antibiotics available for treatment of Gram-positive bacterium such as our chosen methicillin-sensitive strain of *S. aureus* (MSSA), vancomycin presents itself as one of the most aggressive antibiotics available for clinical use and is proven to work very well against methicillin-resistant *S. aureus* (MRSA) while still being effective against MSSA [21, 39, 40, 42, 44]. While toxicity of the drug is often considered a deterrent for its use, vancomycin is only toxic at levels well above the minimum inhibitory concentration for non-resistant *S. aureus* [21, 44]. Regardless, we also assessed whether or not concentrations of vancomycin well above our suspected working levels (up to 200 μg/mL) would be toxic to our W-20-17 cells. Because of the...
many complications involved in performing co-cultures of this nature, we utilized a modified
version of a previously created system in order to establish our model (the details of which are
explained later) [6, 13]. It is important to note that the design of our system is fairly modular,
allowing for the substitution of different cell lines and substances in order to meet the demands
of multiple experimental conditions.

We hypothesize that BMP-2 and VAN will maintain their respective primary functions
on their target cell lines when delivered together in our co-culture system. To test this, cell
viability, proliferation, and differentiation across an array of conditions and time points were
measured. Our findings show that even when delivered together in co-culture, VAN and BMP-2
do not lose their functionality as an antibiotic and growth factor respectively. Moreover, some
evidence suggests that the addition of BMP-2 may reduce the amount of VAN necessary to
inhibit bacterial growth and thus allow for more rapid bone cell proliferation and differentiation.

Materials and Methods

Culture of bacteria. A clinical strain of *S. aureus* (ATCC 6538) was propagated
according to the guidelines provided by the vendor. Briefly, cells were grown in 200 mL of
tryptic soy broth (TSB) in a 1 L Erlenmeyer flask and incubated at 37°C in a humidified
incubator. Once cells reached an OD600 of ~0.5, they were centrifuged (10 min, 4300 g, 4°C)
and re-suspended in 20% glycerol solution. Aliquots of this suspension were then frozen in
liquid nitrogen and stored at -80°C until needed for culture.

Culture of mouse bone cells. W-20-17 cells (ATCC) were propagated according to the
guidelines provided by the vendor. Briefly, cells were grown and maintained in Dulbecco’s
modified Eagle’s medium (DMEM) with 10% FBS, 1% antibiotic/antimycotic mixture, 5 mL of
L-glutamine (200 mM), and sodium pyruvate. This cell line is an ASTM standard to evaluate activity of BMP-2 in vitro. The cells were cultured in an incubator supplied with 5% CO₂ at 37°C. Medium was replaced every three days. To prevent oversaturation of the flask, cultures were periodically passaged prior to the cells reaching high confluence.

**Preparation of VAN and BMP-2 treatments.** Vancomycin treatments were prepared by dissolving dry vancomycin hydrochloride (Acros Organics) in phosphate buffered saline (PBS). Serial dilutions were performed to create stocks with concentrations of 160, 80, 40, 20, and 10 μg VAN/mL [1, 17, 22, 41, 45]. These stocks were then run through a sterile filter and refrigerated until use. BMP-2 stocks (R&D Inc.) were similarly suspended in PBS and stored at -20°C until use.

**Experimental design.** For monocultures, *S. aureus* bacteria were grown in 24-well plates. Appropriate volumes of TSB, VAN, BMP-2, and phosphate buffered saline (PBS) were added to each well such that the final volume was 500 μL (1000 μL for cultures used in ALP and dsDNA assays). Cells from thawed frozen stock were seeded to a final concentration of 10⁵ cfu/mL and incubated at 37°C in a humidified incubator (5% CO₂). Tested final concentrations of VAN were 0, 1, 2, 4, 8, and 16 μg/mL while tested concentrations of BMP-2 were 0 and 100 ng/mL. W-20-17 cells were also grown in monoculture in 24-well plates. Cells were seeded to a final concentration of 15,000 cells per well in DMEM and incubated at 37°C in a humidified incubator (5% CO₂). Tested final concentrations of VAN were 0, 1, 2, 4, 8, and 16 μg/mL while tested concentrations of BMP-2 were 0 and 100 ng/mL. For treatments requiring either zero VAN or BMP-2, the appropriate volume of PBS was added instead. For longer cultures, culture medium and treatments were replaced with fresh medium and the appropriate amounts of each
VAN or BMP-2 treatment every 3 days. All concentrations were kept constant throughout the culture period. 

Bacterial cells used for co-culture were taken from a growing liquid culture. A 125 mL flask containing 10 mL of TSB was inoculated with *S. aureus* bacterium and allowed to grow overnight in a humidified incubator (37°C, 5% CO₂), shaking at 200 rpm. The following day, the culture was centrifuged (10 min, 4300 g, at 4°C), the pellet washed with 10 mL Hank’s Balanced Salt Solution (HBSS), and re-suspended in 10 mL DMEM-p (DMEM, 10% FBS, 0% penicillin, 5 mL of L-glutamine (200 mM), and sodium pyruvate). Co-cultures of *S. aureus* and W-20-17 cells were performed through a series of incubations, washes, and medium changes. First, W-20-17 cells were cultured overnight as described previously. After incubation, culture wells were aspirated and rinsed twice with 500 μL HBSS before replacement of the medium with 480 μL of DMEM-p. Next, the mouse cell cultures were infected with 20 μL of *S. aureus* suspension for an approximate final concentration of 10⁷ cfu/mL, a concentration higher than that used for monocultures in order to aid in the differentiation of treatment groups by using more stringent conditions. This co-culture was then incubated for 45 min at 37°C. Following the 45 min infection period, cell culture wells were aspirated and washed twice with 500 μL HBSS to remove as much extracellular bacteria as possible and incubated at 37°C in 400 μL DMEM-p and 100 μL of a predetermined treatment combination of VAN (0, 1, 2, 4, 8, or 16 μg/mL) and BMP-2 (0 or 100 ng/mL).

**Measurement of metabolic activity.** Metabolic activity was measured using the Promega CellTiter96 AQueous One Solution (MTS) as directed by the supplier. At predetermined time points, the number of viable cells was determined quantitatively according to the manufacturer’s instructions. For monocultures, the metabolic activity of *S. aureus* or W-20-
171 cells was tested separately (in TSB and DMEM mediums respectively). For co-cultures, the
172 metabolic activity of W-20-17 cells was evaluated by culturing a *S. aureus* monoculture (10^7
173 cfu/mL) in parallel with the co-culture (using the same treatments and conditions) and
174 subtracting the monoculture background from the co-culture measurement. To remove as much
175 residual bacteria as possible, culture medium was aspirated, washed twice with 500 μL HBSS,
176 supplied with 400 μL fresh DMEM-p and 100 μLtreatment, and incubated for 30 min before the
177 reagent was added. Assays began with 100 μL of the reagent being added to each well of the 24-
178 well plate. Cultures were then placed in a humidified incubator at 37°C for 1 hour. Lastly, 100
179 μL of each well was transferred to a 96 well assay plate and measured at 490 nm in a microplate
180 reader (TECAN Infinite F50).

**Staining and visualization of cells.** Qualitative analysis of cell cultures was
181 accomplished using Invitrogen Live/Dead cell viability kits as directed by the supplier. Bacteria
182 monocultures were visualized using a Live/Dead BacLight Bacterial Viability kit whereas W-20-
183 17 monocultures and co-cultures were visualized using a Live/Dead Mammalian Cell Viability
184 kit. Photomicrographs of cells were documented using a microscope (Nikon, ECLIPSE TE-
185 2000-U) and processed using MetaVue software (Nikon, MetaVue) after 1, 2, and 3 days of W-
186 20-17 monoculture, 3 days of *S. aureus* monoculture, and 1, 3, and 7 days of co-culture.

**Preparation of cell culture lysate for ALP and dsDNA.** At the designated time, the
188 medium was removed from the cell culture. The cell layers of W-20-17 were washed twice with
189 PBS (pH 7.4) and then lysed with 1 mL of 0.2% Triton X-100 by three freeze-thaw cycles, which
190 consisted of freezing at -80 °C for 30 minutes immediately followed by thawing at 37 °C for 15
191 minutes. The cell lysates were used to determine ALP activity and double stranded DNA.

**Preparation of bacterial cell culture lysate for ALP and dsDNA.** At the designated
time, the samples in each well were mixed and transferred to microcentrifuge tubes and centrifuged for 15 minutes at 10,000 g at 4°C. The supernatant was removed and the pellet was then re-suspended in 1000 µL of 0.2% Triton X-100. Lysis was achieved by performing four freeze-thaw cycles, which consisted of freezing at -80 °C for 30 minutes immediately followed by thawing at 37 °C for 15 minutes. The cell lysates were used to determine ALP activity and double stranded DNA.

**Measurement of ALP activity.** The cell lysates were assayed for the presence of ALP, an important marker for determining osteoblast phenotype. 50 µL aliquots of the cell lysates were sampled and added to 50 µL of working reagent in a 96-well assay plate. The working regent contains equal parts (1:1:1) of 1.5M 2-amino-2-methyl-1-propanol (Sigma), 20 mM p-nitrophenyl phosphate (Sigma), and 1 mM magnesium chloride. The samples then were incubated for 1 hour at 37°C. After incubation, the reaction was stopped with 100 µL of 1N sodium hydroxide on ice. ALP activity was determined from the absorbance using a standard curve prepared from p-nitrophenol stock standard (Sigma). The absorbance was measured at 405 nm using a microplate reader (Bio-Rad model 680). The ALP activity of cells was then calculated by normalizing to double stranded DNA (dsDNA). ALP activity was expressed as nmol/ng.

**Measurement of dsDNA amount.** The cell lysates were examined for double stranded DNA to estimate cell number. 50 µL aliquots of the cell lysates were added in a 96-well assay plate. Each 50 µL of a 1:200 dilution of picogreen (Quant-iTPicoGreen assay kit, Invitrogen) was added to each well and incubated for 5 min in the dark. The assay plate was read at 485 nm excitation and 530 nm emissions using a BioTek FLx800 plate reader. The double stranded DNA content was calculated using a standard curve made by a provided dsDNA standard sample.
Amounts of dsDNA were measured using a picogreen fluorescence assay to standardize ALP production measurements.

**Measurement of interleukin-6 (IL-6) response.** Interleukin-6 production by W-20-17 in co-culture was measured using an Invitrogen IL-6 ELISA kit with minor modifications to the sampling protocol provided by the supplier. Samples tested were prepared from the supernatant of each culture. Briefly, *S. aureus*/W-20-17 co-cultures and W-20-17 monocultures were performed as described previously. After 3 days of incubation, the medium from each well was transferred to individual microcentrifuge tubes and centrifuged for 5 min at 300 g at 4°C. The resulting supernatant was then stored at -20°C until measurement using the ELISA kit. The assay was performed as written in the kit’s instructions. Briefly, the collected samples were added to the assay well plate in duplicate. The prescribed series of reagent additions, washes, and incubations were performed for as instructed. Following the addition of stop solution, absorbance was measured using a TECAN Infinite F50 microplate reader set to 450 nm.

**Statistical analysis.** All data are presented as mean ± standard deviation. For comparing two groups of data, student t-test was performed. For comparing multiple groups of data, one-way ANOVA was performed followed by Tukey’s test. The differences in groups and experimental time points were considered significant if \( p < 0.05 \).

**Results**

**Mouse cell metabolic activity in monoculture.** MTS assays were performed to measure cell metabolic activity at various concentrations of antibiotic VAN (between 0 and 16 μg/mL) and BMP-2 (either 0 or 100 ng/mL) over a three-day period. The results were used to determine
effectiveness at the bacterial concentration tested in co-culture (Fig. 1). W-20-17 cells showed significantly higher metabolic activity at day 3 than at day 1 ($p<0.05$), indicating that the cells were able to grow and proliferate in the presence of VAN. Our results indicate that VAN had little to no measurable effect on overall cell metabolic activity regardless of concentration, BMP-2 presence, or culture duration. Worth noting is that all groups treated with BMP-2 (Fig. 1b) showed lower metabolic activity relative to their BMP-2-free counterparts (Fig. 1a). This suggests that BMP-2 may reduce the overall metabolic activity of W-20-17 cell cultures (possibly by way of reducing cell proliferation in favor of cell differentiation). Live/Dead fluorescence staining also showed that in all treatment groups, W-20-17 cells were viable and maintained their normative spindle-like shapes (Fig. 2). Furthermore, visual assessment indicates an increase in cell proliferation over the three days of culture across all treatments of VAN and BMP-2.

**Mouse cell osteoblastic differentiation in monoculture.** ALP assays were performed to measure osteogenic activity of W-20-17 cells at various concentrations of antibiotic VAN (between 0 and 16 $\mu$g/mL) and BMP-2 (either 0 or 100 ng/mL) over a seven-day period. Our results indicate that VAN had no clear effect on ALP specific activity (ALP normalized to per unit dsDNA) regardless of concentration, BMP-2 presence, or day (Fig. 3). The results suggest that BMP-2 was able to maintain its ability to increase overall ALP activity at all concentrations of VAN. dsDNA levels were measured in order to normalize ALP activity. Results indicate little overall difference in dsDNA levels at day 7, but there is a consistent trend of reduced dsDNA levels at day 5 in the BMP-2 treated groups relative to their BMP-2-free counterparts. Regardless, a statistically significant difference exists between the 0 ng/mL and 100 ng/mL BMP-2 groups in terms of ALP specific activity.
Bacterial metabolic activity in monoculture. MTS assays were performed to measure cell metabolic activity at various concentrations of VAN antibiotic (between 0 and 16 μg/mL) and BMP-2 (either 0 or 100 ng/mL) over a 24-hour period (Fig. 4). Our results indicate that at every measurement time, all treatment groups with ≥1 μg/mL VAN showed highly suppressed \( S. \) aureus growth (for all intents and purposes, \( S. \) aureus metabolic activity was practically zero in these groups). The treatment group with no VAN showed dramatic increases in metabolic activity by 6 hours and immeasurable levels by 24 hours. Overall, our data suggests that even at concentrations of 1 μg/mL, VAN is effective at hindering the growth of our strain of \( S. \) aureus when seeded at \( 10^5 \) cfu/mL. However, because this result proved incapable of distinguishing the efficacy of our treatments, Live/Dead fluorescence staining of \( S. \) aureus as well as all co-culture assays were seeded with bacteria at \( 10^7 \) cfu/mL. At this higher concentration, L/D staining also confirmed \( S. \) aureus lost their viability when treated with at least 2 μg/mL VAN regardless of BMP-2 presence (Fig. 5). Visually, there is a decrease in bacterial cell proliferation within the three days of culture across all treatments of VAN.

Bacterial ALP production in monoculture. Bacterial ALP production was measured to assess whether or not the amount of ALP produced by the tested \( S. \) aureus strain was significant when compared to W-20-17 cells. Therefore, a high concentration of \( S. \) aureus cells (\( 10^9 \) cfu) was cultured in 1 mL of DMEM-p in a shaking incubator for 3 hours. ALP was measured as described previously. Results indicated that \( S. \) aureus strain ATCC 6538 produces about 0.045 nmol ALP per 1 ng dsDNA. This is more than 10-fold less than the ALP specific activity of W-20-17 cultured under similar conditions and is over 100-fold less than the ALP specific activity of W-20-17 cultured with BMP-2. Despite this difference, it cannot be ruled out that ALP production of \( S. \) aureus may affect measurements taken from co-culture. To suppress the
possibly confounding variable of gross bacterial ALP production, ALP specific activity, gross
ALP normalized by dsDNA levels, was used for co-culture assessment rather than raw ALP
measurements.

Metabolic activity of mouse cells in co-culture. MTS assays were performed to
measure cell metabolic activity at various concentrations of VAN antibiotic (between 0 and 16
μg/mL) and BMP-2 (either 0 or 100 ng/mL) over a 7-day period (Fig. 6). L/D staining imaging
was also performed to investigate viabilities of cells (Fig. 7). The results indicate treatments
with greater than 4 μg/mL VAN are effective at stopping bacterial growth in a co-culture over a
7-day period. However, it was also found that a treatment between 1 and 2 μg/mL VAN is the
minimum concentration required to effectively combat bacterial growth after four hours in
monoculture when seeded at 10⁷ cfu/mL (Fig. 5). Important to note is that the MTS assay cannot
distinguish between the metabolic activity of residual bacteria or growing W-20-17 cells when
grown in co-culture. Given the qualitative staining results, it is clear that the large increases in
OD490 in groups treated with less than 8 μg/mL VAN can primarily be attributed to *S. aureus*
growth. Groups with greater than or equal to 8 μg/mL VAN show metabolic activity likely
attributed to the W-20-17. Note that 16 μg/mL VAN is sufficient in both concentrations of BMP-
2 tested. However, a significant decrease in metabolic activity by day 7 is evident in groups
treated with only 8 μg/mL VAN where as there is an increase in the comparable treatment group
with 100 ng/mL BMP-2. This suggests that 8 μg/mL VAN may not be sufficient in retaining W-
20-17 growth unless coupled with 100 ng/mL BMP-2. As shown in Figure 7, the minimum VAN
concentration that allows W-20-17 growth increases at each time point regardless of BMP-2
treatment (red arrows). Staining results showed that with both 0 and 100 ng/mL BMP-2, 2
μg/mL VAN was effective for one day, 4 μg/mL for three days, and 8 μg/mL for seven days.
Qualitatively, treatments with higher concentrations of VAN showed greater W-20-17 proliferation and reduced bacterial growth. When W-20-17 growth is evident, treatments with BMP-2 showed greater growth than those without.

Osteoblastic differentiation of mouse cells in co-culture. ALP specific activity was measured in order to assess the osteoblastic differentiation of W-20-17 cells in response to different vancomycin doses in a co-culture environment over a 7-day period (Fig. 8). Treatments of 4 μg/mL VAN or lower were ineffective at inhibiting bacterial growth (as indicated by low or decreasing ALP specific activity measurements) regardless of BMP-2 concentration. By day 7, ALP specific activities showed a marked increase over day 3 in groups treated with 8μg/mL VAN in combination with 100 ng/mL BMP-2 but not in groups without BMP-2. This suggests that the combination of both BMP-2 and VAN allows for enhanced osteoblastic differentiation when challenged with the parasitic *S. aureus* bacterium in co-culture. ALP specific activity was similar in co-culture with 8 μg/mL and 16 μg/mL VAN as in monoculture. As expected, BMP-2 treated cultures that survived the seven day period exhibited significantly greater ALP production than those without BMP-2, suggesting that BMP-2 maintains its effectiveness in co-culture.

dsDNA amount was primarily measured to assess ALP specific activity. As a whole though, it acts as general measure of cell proliferation. Evident is a progressive increase in dsDNA levels over the seven day culture despite staining evidence suggesting very few W-20-17 cells in the cultures with low VAN concentrations. This is likely the result of bacterial dsDNA influencing measurements. To correct for this, we have reported only ALP specific activity. Because we know that bacteria produce much lower concentrations of ALP than W-20-17 per capita, we can expect that co-cultures with high bacterial concentrations will report low ALP.
specific activities where as those with high numbers of W-20-17 will report values closer to that of the W-20-17 monoculture.

**Interleukin-6 production of cells in co-culture.** Interleukin-6 (IL-6) is a protein found to be secreted by human and mouse osteoblasts at elevated levels in response to infection by bacteria (specifically *S. aureus*) [6]. Our results indicate that IL-6 production was only significantly increased with exposure to bacteria in groups with 16 μg/mL VAN treatment (Fig. 9). Treatments less than or equal to 1 μg/mL VAN displayed a reduction in the amount of IL-6 produced. This is likely attributed to a decrease in the raw number of W-20-17 in culture. Treatments from 2-8 μg/mL VAN showed no significant change in IL-6 production. Groups treated with 16 μg/mL VAN showed an increase in the amount of IL-6 produced. This suggests a greater number of cells combating infection by *S. aureus*.

**Discussion**

The goals of this study were to establish an in vitro co-culture model system to better represent the in vivo scenario of dual drug delivery and to gain a better understanding of the interactions between two specific compounds of choice: vancomycin antibiotic and bone morphogenetic protein 2. Briefly, we treated mouse bone marrow stromal cells (W-20-17) and *S. aureus* cells (ATCC 6538) with various concentrations of VAN (0-16 μg/mL) and BMP-2 (0-100 ng/mL) in both mono- and co-culture. In order to assess cell proliferation, differentiation, morphology, and inflammation response, an array of assays were performed for each treatment group across cultures ranging from 1 hour to 7 days. We selected W-20-17 because it has been used for an ASTM (F2131) standard test for in vitro biological activity of BMP-2. *S. aureus* was chosen for both its clinical origin as well as this species’ high prevalence in bone graft failures [2,
If desired, modification of the system we establish here could accommodate for testing of other antibiotic or growth factor compounds across a wide array of mammalian/bacterial cell co-cultures. Similarly, the utilization of other antimicrobials such as silver ions should also be considered in order to complement the activities of substances such as VAN and BMP-2 [43, 51].

With regard to the efficacy of VAN antibiotic, we found that in co-culture, VAN behaved differently than expected based on our monoculture findings. Staining results showed that in co-cultures with both 0 and 100 ng/mL BMP-2, 2 μg/mL VAN was effective for one day, 4 μg/mL for three days, and 8 μg/mL for seven days. This suggests that while bacteria were kept in check by some concentrations of VAN early on, concentrations below 8 μg/mL were not effective at reducing bacterial growth for extended periods of time. When compared to S. aureus monoculture L/D staining, co-culture MTS results showed that in co-culture, a higher dosage of VAN (8 μg/mL vs 2 μg/mL) was necessary to effectively inhibit bacterial growth than that same treatment group in monoculture (Fig. 5 and 6). By day 7, it would be expected that the S. aureus monocultures treated with at least 2 μg/mL VAN would continue to show little to no bacterial growth, but the accuracy of this approximation requires further testing. Still, this suggests that monocultures may not be a representative model for antibiotic dose dependence in our co-culture model. Furthermore, this suggests that the co-culture environment may be limiting the efficacy of VAN. It is proposed that internalization of the S. aureus by the osteoblasts allows for the bacteria to evade any vancomycin in solution, thus reducing the efficacy of the drug [13]. However, further investigation is required to elucidate this mechanism, either through mechanistic study or through testing of alternative cell lines and compounds.
In both mono- and co-culture, we found that for all concentrations tested (0-16 μg/mL), VAN had no evident effect on the growth of mouse bone cells. Even at concentrations of up to 200 μg/mL VAN (data not shown), we found that the antibiotic had no detrimental effect on W-20-17 proliferation, differentiation, or morphology. This result stands true in both treatments with only VAN and in those that also received BMP-2. Clearly, VAN is a good candidate as the antibiotic of choice in our proposed dual delivery system in that it is effective against \textit{S. aureus}, the primary offender in bone graft failure, and has no evident toxicity to host osteoblasts at effective concentrations. Furthermore, our results are consistent with the available literature [36].

The second prong of our proposed dual delivery system, BMP-2, showed that in both mono- and co-culture it is capable of creating a significant increase in ALP production (an early marker of osteoblastic differentiation). In our qualitative L/D staining assessment, cultures treated with 100 ng/mL BMP-2 showed better growth than those without BMP-2 treatment. This qualitative result is backed by the quantitative MTS and ALP assay results. ALP specific activity was used to differentiate between bacterial proliferation (low ALP specific activity) and mBMSC proliferation (high ALP specific activity). On average, treatment with BMP-2 resulted in a statistically significant increase in ALP specific activity (from less than 0.5 nmol/mL to as much as 2.5 nmol/mL). This pattern is also evident in co-culture, suggesting that BMP-2 maintains its functionality in both mono- and co-culture. BMP-2 showed little to no effect both qualitatively and quantitatively on the monocultures of ATCC 6538 \textit{S. aureus} within the 24-hour time frame tested. This remained true for all concentrations of VAN tested, further positing that BMP-2 can maintain its positive effect on mouse bone cells while generating little to no positive effect on bacterial cells.
While BMP-2 and VAN behaved as expected when delivered in co-culture, their combined effect on both the W-20-17 and *S. aureus* has, to our knowledge, never been established before despite their dual delivery previously having been explored [18, 28, 47]. It is important to emphasize that across all concentrations of VAN tested, BMP-2 had no measurable detrimental effect on the drug’s action against the Gram-positive bacterium. In fact, we found that some assays indicated that in treatments with BMP-2, a lower concentration of vancomycin was able to achieve almost complete suppression of bacterial growth compared to treatments without BMP-2 (8 μg/mL VAN in co-cultures with BMP-2 versus 16 μg/mL in co-cultures without BMP-2). The true mechanism behind this result cannot be determined due to the limitations of our assays, but we believe that this may be a result of BMP-2 conferring a competitive advantage upon the mBMSCs against the bacterium. It is suggested that BMP-2 allows for improved cell growth and differentiation by day 7, thus allowing for bone cell growth to outpace the bacteria’s capacity to infect and proliferate. Because this effect has not been observed before, further investigation into this interaction is necessary. If found to be true, this type of interaction would be suspected to be observed in treatments with other combinations of antibiotics and growth factors capable of maintaining function when delivered together. Such a finding could very well bring the study of pro-host/antimicrobial compound dual delivery to the forefront of anti-infection research.

Here we begin an attempt to bridge the two major avenues of study in bone tissue repair: accelerated growth and improved treatment success rates. While each of these concepts has already been implemented into standard clinical care in the form of antibiotic and bone growth factor delivery, there still remains a need for refinement in their execution. This is the first of many studies aimed at creating a streamlined dual delivery system that is capable of delivering
both antibiotic drugs and bone growth factors to achieve those aforementioned goals. We have established a mBMSC/S. aureus co-culture model system that is aimed at mimicking the \textit{in vivo} condition of bone graft infection and giving consistent, predictable results. From this model system, we have begun to characterize the interactions of two drugs of interest: VAN antibiotic and BMP-2 growth factor. Our findings indicate that individually, they maintain their expected functionality as an antibiotic and growth factor respectively, but, in combination, their combined effect may create a lower demand on the toxic, expensive use of VAN. As a whole, these results pave the way for future study in the dual delivery of antibiotics and growth factors. We plan to begin testing both delivery timing and more material-focused studies regarding the actual mechanistic approach to delivering said components using the co-culture model system established here.

\textbf{Acknowledgments}

We would like to acknowledge the supports from DOD W81XWH-10-1-0966 and W81XWH-10-2-0010, Airlift Research Foundation, Wallace H. Coulter Foundation, NIH R01AR057837 from NIAMS, and NIH R01DE021468 from NIDCR.
References


PLGA composite grafts to induce bone repair in grossly infected segmental defects. Biomaterials. 31(35):9293-300.

Figure legends

**Figure 1.** Measure of metabolic activity of W-20-17 treated with 0-16 μg/mL VAN in combination of (a) 0 ng/mL BMP-2 and (b) 100 ng/mL BMP-2 over 3 days using MTS colorimetric assay. Higher OD490 values indicate greater metabolic activity. The initial cell density was 15,000 cells per well for 3 days. The absorbance is expressed as a measure of the cell viability via cell culture media for 3 days of incubation. Each value represents the mean ± SD (n = 3). * denotes significant difference compared with 1 day of culture (p<0.05). ** denotes significant difference between different time points (p<0.05).

**Figure 2.** Live/Dead fluorescence staining of W-20-17 treated with 0-16 μg/mL VAN and either (a) 0 ng/mL BMP-2 and (b) 100 ng/mL BMP-2 in monoculture in DMEM medium. Photos were taken at 100 X magnification after up to three days of culture. Green indicates live bone cells and red indicates dead cells.

**Figure 3.** Measure of early osteoblastic differentiation of W-20-17 treated with 0-16 μg/mL VAN in combination of (a) 0 ng/mL BMP-2 and (b) 100 ng/mL BMP-2 in monoculture using an ALP assay. The ALP activity was determined at 5 and 7 days of cultures and normalized for the dsDNA content. ALP activity is expressed as nmol/ng. Each value represents the mean ± SD (n = 3). * denotes significant difference compared with 1 day of culture (p<0.05).

**Figure 4.** Measure of metabolic activity of *S. aureus* treated with 0-16 μg/mL VAN in combination of (a) 0 ng/mL BMP-2 and (b) 100 ng/mL BMP-2 over 24 hours using MTS colorimetric assay. The initial concentration of *S. aureus* was 10^5 cfu/mL. Higher OD490 values indicate greater metabolic activity. The absorbance is expressed as a measure of the cell viability...
via cell culture media for 24 hours of incubation. Each value represents the mean ± SD (n = 3). * denotes significant difference compared with 2 hours of culture (p<0.05).

**Figure 5.** Live/Dead fluorescence staining of *S. aureus* treated with 0-16 μg/mL VAN in monoculture and 0-100 ng/mL BMP2 in TSB medium. Photos were taken at 100 X magnification after four hours of culture. Green indicates live bacterial cells whereas red indicates dead cells. Evident is a visual decrease in cell viability over the three days of culture across all treatments of VAN.

**Figure 6.** Measure of metabolic activity of W-20-17 in co-culture treated with 0-16 μg/mL VAN in combination of (a) 0 ng/mL BMP-2 and (b) 100 ng/mL BMP-2 over 7 days using MTS colorimetric assay. Higher OD490 values indicate greater metabolic activity. The initial cell density was 15,000 cells per well. The absorbance is expressed as a measure of the cell viability via cell culture media for 7 days of incubation. Each value represents the mean ± SD (n = 3). * denotes significant difference between different time points (p<0.05).

**Figure 7.** Live/Dead fluorescence staining of *S. aureus* and W-20-17 co-culture treated with 0-16 μg/mL VAN in combination of (a) 0 ng/mL BMP-2 and (b) 100 ng/mL BMP-2 in DMEM-pA medium. Photos were taken at 100 X magnification after 1, 3, and 7 days of culture. Green indicates live cells whereas red indicates dead cells. Smaller, spherical shapes are indicative of bacteria while larger, more irregular shapes are W-12-17. Red arrows indicate effective dosage of VAN.

**Figure 8.** Measure of early osteoblastic differentiation of W-20-17/*S. aureus* co-culture treated with 0-16 μg/mL VAN in combination of (a) 0 ng/mL BMP-2 and (b)100 ng/mL BMP-2 using an ALP assay. Measurements were taken after 1, 3, and 7 days in cultureand normalized for the
dsDNA content. ALP activity is expressed as nmol/ng. Each value represents the mean ± SD (n = 635). * denotes significant difference between different time points (p<0.05). VAN treatments had a significant effect on ALP specific activity. Higher concentrations of VAN were correlated with higher ALP specific activities, suggesting that most of the activity was being generated by mBMSCs in culture as opposed to S. aureus which have a significantly lower ALP specific activity (see Fig. 7). Treatment with 100 ng/mL BMP-2 yielded significantly higher ALP specific activity than treatment without BMP-2. These results suggest that in co-culture, both VAN and BMP-2 are able to maintain their respective functions.

**Figure 9.** Measurement of W-20-17 inflammation response when in co-culture with S. aureus and treated with 0-16 μg/mL VAN and 100 ng/mL BMP-2 using an IL-6 ELISA kit. * denotes significant difference between mono culture and co-culture (p<0.05).