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CLINICALLY RELEVANT DOSAGE OF VANCOMYCIN DOES NOT NEGATIVELY IMPACT PERIOSTEUM DERIVED OSTEOBLAST PRECURSOR CELLULAR FUNCTIONS

By

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Submitted in partial fulfillment of the requirements for the degree

Master of Biology

Department of Biological Sciences of Seton Hall University

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APPROVAL FOR SUCCESSFUL DEFENSE

Alexis Hernandez has successfully defensed and finalized the text of his master's dissertation for the M.S. during this **Fall Semester 2021.**

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Abstract

Surgical site infections (SSI) can develop post-operatively and carry significant clinical and financial implications. SSI can carry a cost of up to $30,000 per case, as well as an estimated 6 day longer hospitalization. Patients with Type II Diabetes (DM) have an increased susceptibility to infection and suffer from poor bone healing overall. Therefore, diabetic patients who have undergone orthopedic surgery risk both an increased chance of developing an SSI as well as suboptimal bone healing. Vancomycin and other antibiotics have traditionally been used prophylactically to prevent infection, however the effect of vancomycin on bone healing in a diabetic population has not been well documented. Periosteum derived osteoblasts were treated with BMP-2, and vancomycin concentrations of 0 ug/mL, 5 ug/mL, 50 ug/mL, 500 ug/mL, and 5,000 ug/mL. The purpose of this work was to examine the effects, if any, that vancomycin had on periosteum derived osteoblast precursor functions including calcium deposition, osteoblast differentiation, and cell viability. Continuously dosed osteoblasts at a concentration of 5,000 ug/mL had a 3-fold decrease in calcium deposition when compared to the BMP-2 treated group ($P<0.005$). In addition, cell proliferation was significantly decreased by 33% at a dosage of 5,000 ug/mL 24 hours post treatment ($P<0.005$). Finally, a 3-fold decrease in osteoblast differentiation was seen when continuously dosed at 5,000 ug/mL ($P<0.005$). However, these effects seen at 5,000 ug/mL are well above the mean inhibitory concentration for resistant MRSA infections of 16 ug/mL. Therefore, clinically appropriate dosages of topical vancomycin can be used prophylactically in the diabetic patient, without concern for further inhibiting bone healing post-repair

Introduction

The Importance of Studying Antibiotics as Treatment for SSI Infections

Surgical site infections (SSI) are costly post-operative complications with negative financial and clinical consequences. Financially, treatment of SSI is estimated to cost between $400 and $30,000 per case in the United States (Urban 2006). Clinically, surgical site infections have consequences including prolonged hospitalization, extended recovery time, and increased cost of post-operative care (Qadir et al., 2021). Kirkland et al., performed a follow up study of patients admitted to a 415-bed community hospital and found that patients who developed SSI had an approximately 6 days longer stay with an estimated $3,000 in additional costs (Kirkland et al. 1999). The importance of using antibiotics as a treatment cannot be understated as a meta-analysis done by AlBuhairan et al, found that antibiotic prophylaxis reduced the relative risk of infection by 81% to those untreated individuals following joint arthroplasty (AlBuhairan et al., 2008). Given the increased implications from SSI and the effectiveness of antibiotic prophylaxis, determining effective and safe usage of these antibiotics is of utmost importance.

Stages of Bone Fracture Healing

The understanding of bone repair is important, not only for the treatment of minor fractures as a result of injuries, but also for effective management of those with comorbidities affecting bone healing (Office of the Surgeon General, 2004). Bone fracture healing can be separated into four distinct steps: hematoma formation, fibrocartilaginous formation, bony callus formation, and bone fracture remodeling.

In humans, bone healing begins with the formation of a hematoma immediately following fracture or injury and lasts about 5 days. The initial trauma to the area will rupture the blood vessels and periosteum around the site and cause a collection of blood, or hematoma, to form around the fracture. This hematoma will form the initial framework for subsequent repair. It is during this period in which a multitude of proinflammatory cytokines including tumor necrosis factor alpha (TNF-alpha), bone morphogenic proteins (BMPs), and interleukins will be upregulated (Sheen and Garla 2021). It is these factors that will recruit inflammatory cells and promote angiogenesis or the formation of new blood vessels. (Marsell and Einhorn 2011). IL-6 will specifically stimulate angiogenesis as well as vascular endothelial growth factor (VEGF) which will drive the next step, fibrocartilaginous formation.

The next step of the fracture healing process, taking place on days 5 to 11 post injury, involves the recruitment of mesenchymal stem cells (MSC) (Marsell and Einhorn, 2011). MSCs recruited to the fracture site proliferate and differentiate into the cells needed for chondrogenesis, specifically chondrocytes (Granero-Moltó et al., 2008). In addition, the disruption of the local vasculature results in a hypoxic state, which aids in the differentiation of the recruited MSCs towards the chondrogenic, cartilage building, pathway and results in the formation of a cartilaginous callus. (Loi et al., 2016). This cartilaginous tissue provides a microenvironment that favors chondrocyte differentiation, while preventing osteoblast function (Claes et al., 2012). Additionally, the low oxygen state promotes revascularization and neo-angiogenesis at the fracture site. Throughout this process, further blood vessels will form into the callus allowing for the migration of

additional MSCs. The recruitment of these stem cells will result in the formation of fibrin-rich granulation tissue and subsequently lead to the formation of a soft callus.

The soft callus, at this time made of cartilage, will then undergo endochondral ossification and be replaced by bone from days 11 to 28. The replacement of cartilage first begins with the resorption of the soft callus and subsequent calcification by osteoblasts and osteoclasts. By the end of these steps the soft callus will be replaced by a hard bony callus and closure of the fracture (Marsell and Einhorn 2011). The last, and longest step, of bone fracture healing is known as bone remodeling. The hard callus itself is not mature bone and further bone resorption by osteoclasts and bone deposition by osteoblasts will replace the callus with compact bone and the edges of the callus will be replaced by lamellar bone. Most notably, this step can last anywhere from months to years to fully regenerate the bone.

Inflammation And Its Relationship to Poor Bone Fracture Healing

The immune system and its components make critical chemical messengers that aid in the stimulation of osteogenesis when an external trauma triggers acute inflammation. However, in a chronically inflamed environment, including disease states such as diabetes mellitus (DM), inflammation can disrupt osteogenesis (Claes et al, 2012). It is important to note that the fracture healing processes described prior are under ideal conditions, with an appropriate inflammatory response. Problems arise when chronic inflammatory disease states caused by rheumatoid arthritis (RA), chronic obstructive pulmonary disease (COPD), diabetes mellitus (DM) and system lupus

erythematosus (SLE) coexist during bone fracture (Claes et al, 2012). Chronic inflammation results in the upregulation of TNF-alpha and NF-kβ signaling pathways. This in turn increases osteoclast resorption, which contributes to excessive bone loss and poor bone fracture healing (Maruyama et al., 2020). Data also demonstrates that increased inflammatory state will lead to the amplified recruitment of neutrophils into the fracture site, which stimulates chondrogenesis, but inhibits osteogenesis (Bastian et al. 2018).

Type II DM, SSI Relationship, And Impaired Bone Healing

Type II DM is often associated with a higher risk of infection for a variety of different surgical procedures including cardiothoracic, gastrointestinal, and most notably orthopedic. Olsen et al. performed a retrospective case-control study of patients following orthopedic surgery and found that diabetic patients, specifically those with elevated serum glucose levels indicative of diabetes, had the highest independent risk of developing a SSI, as indicated by an odds-ratio of 3.5 (Olsen et al., 2008). Latham et al. additionally found that diabetic patients, both diagnosed and undiagnosed, had an increased association with the development of surgical site infections (Latham et al., 2001). These surgical site infections specifically inhibit callus formation and instead causes the formation of fibrous tissue. This in turn will lead to decreased mechanical stability and overall poor fracture healing (Croes et al. 2019). Interestingly, undetected non-union of fractures have been assumed to have been caused by low grade infections (Andrzejowski and Giannoudis 2019).

Vancomycin As a Treatment For SSI

Postoperative infection following orthopedic surgery is a concern that is most mitigated using topical antibiotics. Many times, the antibiotic may be either topical vancomycin, tobramycin, or a combination of both (Owen et al., 2017). Vancomycin prevents bacterial growth by inhibiting bacterial cell wall production, specifically the polymerization of peptidoglycans (Watanakunakorn 1984). It is especially effective in preventing infections by gram-positive bacteria, and more importantly methicillin-resistant Staphylococcus aureus (MRSA) (Patel et al., 2020.) As per the CDC, MRSA strains with decreased susceptibility to vancomycin have a vancomycin MIC of 4-8 μg/mL, whereas resistant strains have a vancomycin MIC greater than 16 μg/mL Versalovic et al., 2011). Although systemic use of vancomycin in preventing bacterial infections is common, the use of topical vancomycin powder in preventing surgical site infections (SSI) after orthopedic procedures has not yet been approved by the U.S Food and Drug Administration (Takahashi et al., 2018). However, research does support the local administration of vancomycin. Kanj et al. found that orthopedic surgery patients who receive local vancomycin were 4 times less likely to develop a deep postoperative wound infection, when compared to those untreated patients (Kanj et al., 2013). In a retrospective review of 272 patients, with groups consisting of standard intravenous antibiotics and topical vancomycin, 6 patients from the standard treatment group developed an infection, compared to none in the vancomycin group. With a $P=0.0027$ it was found that there was indeed a statistical difference in treatment types (Yan et al., 2014). In another retrospective analysis, the outcomes of fractures treated with and

without topical vancomycin was assessed. It was found that none of the 35 patients (0% infection rate) treated with vancomycin developed an infection, compared to 58/548 (10.6% infection rate) patients not treated with vancomycin, thereby providing further support for the use of topical antibiotics (Qadir et al., 2021). Although the efficacy of topical antibiotic powder to reduce infection is well documented, it is less clear whether it negatively impacts osteogenesis.

Vancomycin and Osteogenesis

Current data examining vancomycin treatment and osteogenesis *in vitro* are unclear. Yu et al's studied bone marrow stromal cells (BMSCs) incubated in vancomycin, tobramycin, and both over 7 days. The study found the local administration of vancomycin (14.28 ug/mL), tobramycin (28.57 ug/mL) or the combination of the two (vancomycin 14.28 ug/mL, tobramycin 28.57 ug/mL) did not negatively impact cell viability or proliferation. (Yu et al., 2020). Edin et al. cultured MG-63 human osteosarcoma cells in vancomycin at concentrations of 0, 10, 100, 1000, and 1000 ug/mL with analysis at 0, 24, and 72 hours of treatment. Their results indicated a significant decrease in cell growth at a vancomycin concentration of 10,000 ug/mL at the 24- and 72-hour time points. However, increases in cell death were not noted at the lower concentrations. (Edin et al., 1996). In contrast, Braun et al. explored the effects of human primary osteoblasts at concentrations of 10, 100, 200, 500, 1000, and 2000 ug/mL at days 1, 3, 5, and 10. They found that human primary osteoblasts had decreased proliferation on day 1 at a concentration of 2 mg/mL. However, increasing concentrations beginning from 200 mg/mL had a 20-30% decrease in cell viability (Braun et al., 2020).

In vivo studies focused on the effect of vancomycin and bone are limited. One in vivo study examined posterolateral lumbar spinal fusion of 36 rats divided into three treatment groups including: no vancomycin, standard dose vancomycin (14.3 mg/kg) and high dose vancomycin (143 mg/kg). They found that there were no significant differences in spinal fusion across both treatment groups when compared to the control. In addition, microCT data demonstrated no differences in volume of newly generated bone of the vancomycin groups (both standard and high dose) when compared to control. This data provided compelling evidence that vancomycin treatment may not negatively affect osteogenesis (Mendoza et al, 2016). In a clinical setting examining systemic effects, Owen et al., performed a retrospective study to determine the incidence of SSI after pelvic ring and acetabulum fracture surgery. They found that the risk of infection in the patients treated with 1g vancomycin and 1.2g tobramycin powder had a 4.2% risk of infection, significantly less than that of the untreated group which was 14.5% (Owen et al, 2017).

The importance of effectively reducing SSI in Type I DM cannot be understated as improvement in this area can both improve patient and economic outcomes (Potter, 2018). Our research aims to determine clinically relevant dosages of vancomycin that will not inhibit osteoblast cellular functions. More specifically, we hypothesized that the vancomycin doses that are effective at treating infection (<16ug/mL) will not negatively impact osteoblast precursors derived from Type I DM rats.

Materials and Methods

Bone Marrow Cell Isolation

The femora of 3-month-old BB Wistar Type I DM rats were resected. The distal and proximal ends of the femurs were cut, to expose the bone marrow. A 16G needle was inserted into one end of the femur and the marrow was subsequently flushed using 5 mL of α-MEM, supplemented with 10% v/v FBS and 0.5% v/v penicillin-streptomycin. The femur was inverted and flushed with an additional 5 mL of PBS. The cells were cultured undisturbed at 37°C, 5% CO_2 for 48 hours and then the adherent cells were sub-cultured for use in experiments.

Periosteum Cell Isolation

The tibia and fibula of 3 month old BB Wistar Type I DM rats were resected. Muscle and other tissues were carefully cut from the isolated bone. The periosteum was then gently scraped off and placed cambium side down onto a 100 mm culture dish. PBS supplemented with 1% v/v fungizone, and 0.5% v/v penicillin-streptomycin was used to wash the samples three times. Afterwards the periosteum samples were placed onto a 100mm culture dish without media and incubated at 37°C, 5% CO_2 for 5 mins to allow the tissue to adhere to the plate. Once adhered, the isolated periosteum was cultured in α-MEM supplemented with 10% v/v FBS, 0.5% v/v penicillin-streptomycin, 1% v/v fungizone at 37°C, 5% $C0_2$ [25]. Media was changed every 2 days until migration of cells from the periosteum samples was visualized. The periosteum remnants were then removed, and the cells were cultured until confluency, approximately every 7 days. Cells were passaged using trypsin to remove the adherent cells and sub-cultured.

Osteogenic Differentiation and Treatment Groups

Rat bone marrow cells and periosteum cells were cultured α-MEM supplemented with 10% v/v FBS, 0.5% v/v penicillin-streptomycin, 1% v/v fungizone, 10 mM ß-glycerophosphate and 100 µM L-ascorbic acid to induce osteogenic differentiation [26]. After the cell cultures reached confluence, vancomycin was added to the osteogenic media at 0, 5, 50, 500, or 5,000 µg/mL. Single dose (SD) cultures were treated with vancomycin for 48 after confluence and then returned to osteogenic media without vancomycin till the experimental endpoint. Once confluent, continuous dosing (CD) cultures were switched into and maintained in vancomycin supplemented osteogenic media until the experimental endpoint. BMP-2 (10 ng/mL) was added to osteogenic media as a positive control and used for an alkaline phosphatase assay, alizarin red staining and MTT assay.

MTT Assay

Periosteum cells were seeded, in triplicate, onto a 96 well dish at a seeding density of 5.0 $x10^4$ cells/mL in standard culture medium and allowed to attach overnight. After confirming adherence, the media was replaced with vancomycin at increasing concentrations (0, 5, 50, 500, 5000 ug/mL) and cells were incubated at 37°C and 5% CO_2. After 24-hours of treatment, media was aspirated from each well and replaced with MTT solution and incubated at 37°C, 5% CO_2 for 2 hours. Three volumes of DMSO were added to each well and the plate was incubated on an orbital shaker for 30 minutes at room temperature. The plate was visualized under the microscope to ensure all purple

crystals dissolved into the DMSO solvent. The absorbance of each well was detected on a spectrophotometer (Thermo-Fisher Waltham, Massachusetts) at an O.D of 590nm.

Alizarin Red Staining

Calcium deposition in the cell cultures was measured after 14- and 28-days using Alizarin Red S staining in triplicate. Media was carefully aspirated from each culture and the cells were washed 2 times with PBS before fixing in 0.5 mL of 10% formaldehyde for 15 minutes at room temperature. The formaldehyde was removed, and the fixed cells were washed 2 times with PBS before adding 1 mL of 40 mM Alizarin Red Stain (ARS). Cultures were incubated at room temperature for 20 minutes with gentle shaking. The stained cultures were washed with water 4 times. To each culture well, 0.8 mL of 10% acetic acid was added and incubated at room temperature for 30 mins with gently shaking. The culture extract was transferred to a microcentrifuge tube and any remaining, adherent cells were scraped from the culture well and added to the microcentrifuge tube. The samples were then vortexed for 30 seconds, heated at 85°C for 10 mins, and transferred to ice for 5 minutes. Debris was removed by centrifugation (20,000 g for 15 minutes). An aliquot of the supernatant (500 µL) was transferred into a new microcentrifuge tube to which 200 µL of 10% (v/v) of ammonium hydroxide was added. Aliquots of the neutralized extract (150 µL) were transferred into a 96-well plate and the absorbance was measured on a spectrophotometer (Thermo-Fisher Waltham, Massachusetts) at an O.D of 405 nm.

Alkaline Phosphatase Assay

Alkaline phosphatase activity was measured in media aliquots using the colorimetric para-nitro-phenyl phosphate (pNPP) assay with reagents obtained from Sigma-Aldrich (St. Louis, MO). Media was collected from cell cultures at 7, 14, 21, and 28 days in triplicate and stored at -20°C before measuring alkaline phosphatase activity.

Results

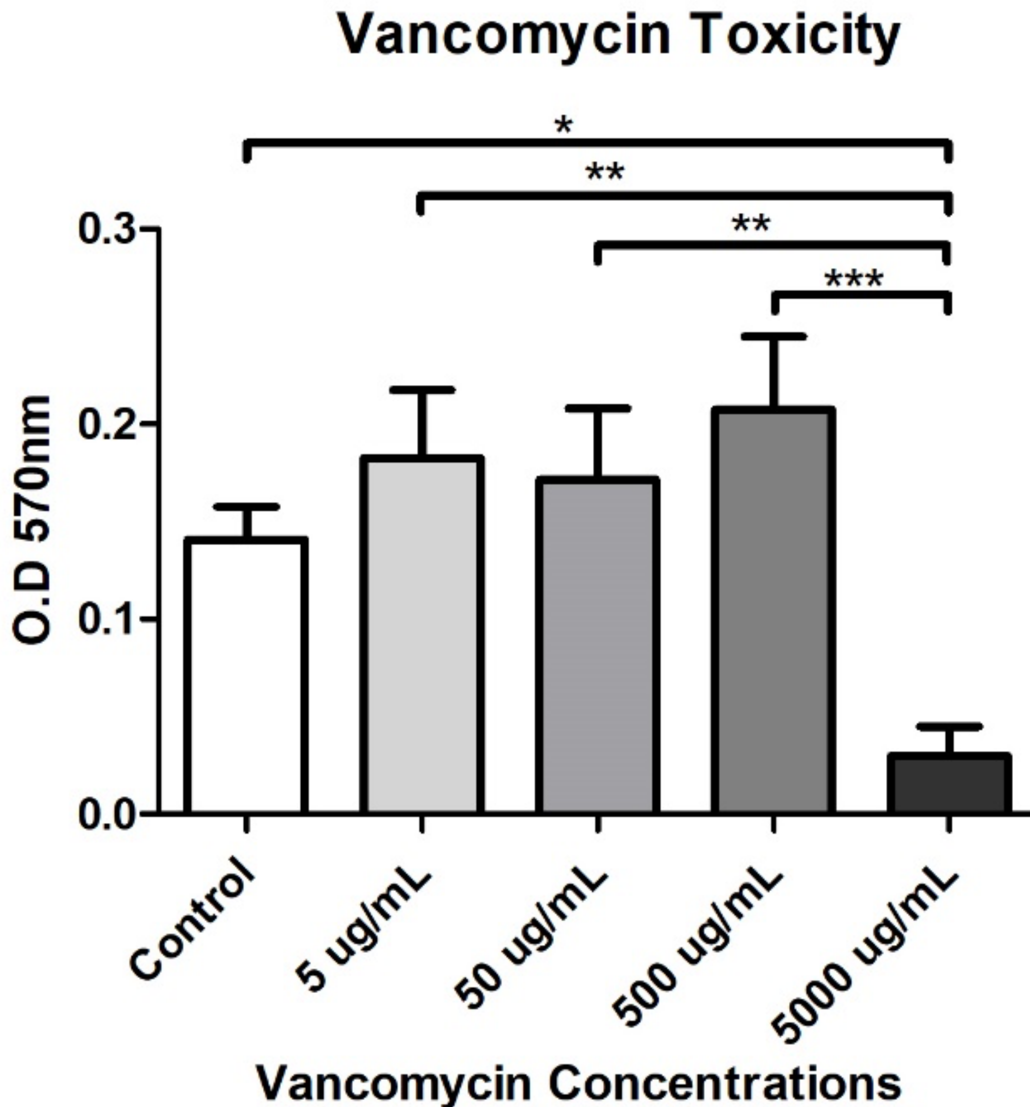
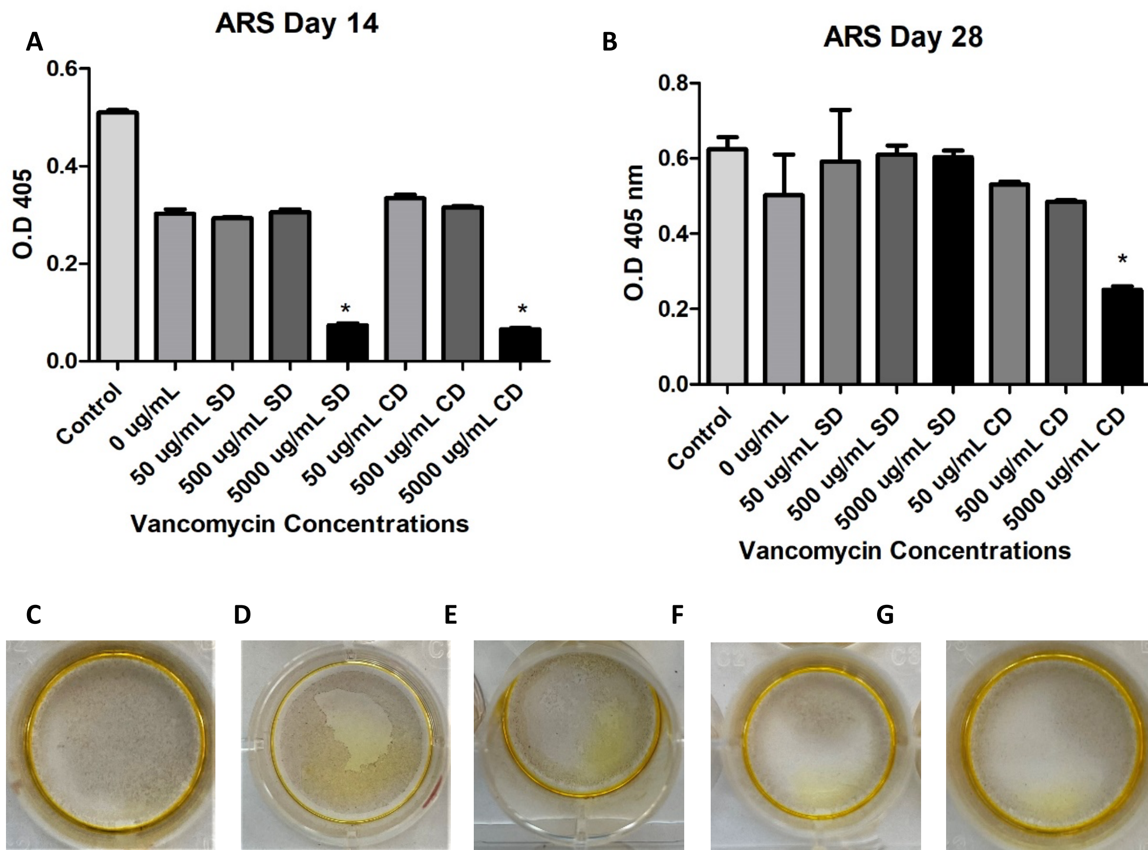


Figure 1. **Periosteum Cell Viability via MTT Assay**. As indicated above there was statistically significant cell toxicity in the samples treated with a dosage of 5000 ug/mL when compared to control and lesser dosages. There is no difference in each of the treatment groups against one another, or when compared to the untreated, control, group. Statistical significance was indicated with a $P<0.005$.

Vancomycin's Effect on Cellular Proliferation

Periosteum proliferation was significantly decreased by 33% at a dosage of 5,000 ug/mL 24 hours post treatment, when compared to 0, 50, and 500 ug/mL doses (Figure 1). A slight increase in proliferation was seen with increasing vancomycin dosages (5ug/mL to 500 ug/mL) when compared to the untreated group although it is found to not be statistical significant. This therefore indicates that at a concentration of 5,000 ug/mL, vancomycin is acutely toxic to the cells and decreases overall proliferation.



(Hernandez, 2021).

Figure 2. **Two- and Four-Week Alizarin Red Staining of Periosteum Derived Osteoblasts.** Calcium deposition levels were observed (Panels C through G) and quantified via spectrometry reading at 405nm at week 2 (Panel A) and week 4 (Panel B). Quantification of the staining reveals a statistically significant ($P<0.005$) decreased calcium deposition in vancomycin dosages of 5000 ug/mL SD and CD. Panel C demonstrates positive control with BMP-2 treatment. Panel D demonstrates growth with osteogenic media, but no treatment. Panels E-G are from continuously dosed samples. All treatment types, SD and CD, had decreased calcium deposition values when compared to the control treated group. However, no difference was noted between the control and 50 ug/mL and 500 ug/mL treatment groups.

Vancomycin's Effect on Calcium Deposition

Alizarin red is stain used to indicate calcium deposits in culture, and in this experiment was used to detect overall calcium deposition, with more visible staining indicating increased calcium deposition by the differentiated osteoblasts. As expected, the positive control, BMP-2 treatment resulted in a 40% significant increase in calcium deposition when compared to 0 ug mL, 50 ug/mL, 500 ug/mL, and 5,000 ug/mL (Figure 2, $P<0.005$). At day 14 there was a 3-fold decrease in calcium deposition from both the single and continuously dosed 5,000 ug/mL treatment groups ($P<0.005$). However, at day 28, there was only a 3-fold decrease in calcium deposition at CD 5,000 ug/mL vancomycin. Interestingly, the SD 5,000 ug/mL vancomycin treated group, which initially demonstrated an 80% decrease in calcium deposition on day 14, had recovered to levels like the control and remaining treatment groups by day 28. Overall, there was an increase in calcium deposition seen in all the treatment groups, including the CD 5,000 ug/mL group between days 14 and 28.

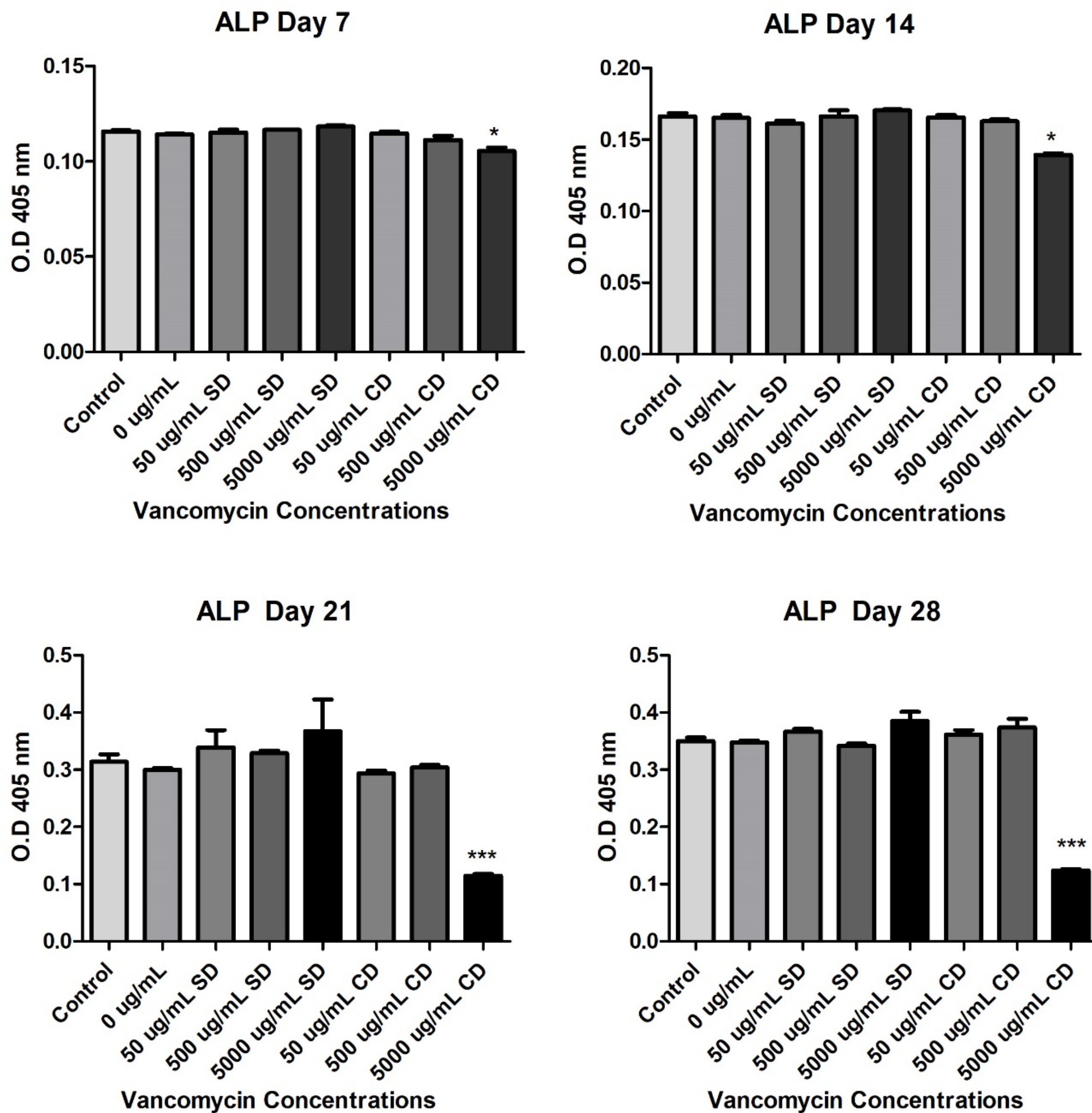


Figure 3. **Alkaline Phosphatase Activity Following Treatment with Vancomycin**. Alkaline phosphate activity at days 7, 14, 21, and 28. At all-time points there is a statistically significant decrease in ALP activity when continuously dosed (CD) at a concentration of 5,000 ug/mL. Lower concentration groups, both continuously dosed and single dosed, demonstrate no decrease in activity. Statistical significance is defined as P<0. 005.Similar ALP levels are noted throughout control samples, with BMP-2, untreated, and treated samples up to 500 ug/mL dosages, including single dose (SD) and CD.

Vancomycin's Effect on Osteogenic Differentiation

Alkaline phosphatase (ALP) has generally been regarded as an early marker for assessment of osteoblast differentiation, with increased detection of ALP indicating increased differentiation. ALP can be regarded as a marker since it is a byproduct of osteoblast activity and therefore indicative of active differentiated osteoblasts. Periosteum cells continuously dosed at a concentration of 5,000 ug/mL showed a statistically significant decrease in osteogenic differentiation, as assessed through decreased ALP activity ($P<0.005$, Figure 3). Single dosed samples at 5,000 ug/mL did not reveal decreased ALP activity when compared to the controls or lower dosages throughout the 28-day timepoint. At day 21 and 28, a 3-fold decrease in ALP activity at 5,000 ug/mL was found when compared to lower dosages and control ($P<0.005$). SD and CD periosteum cells from 5 to 500 ug/mL maintained similar values when compared to the control treatment group throughout all time points. It is important to note however, that although minimal, there is still some detectable levels of ALP in the 5,000 ug/mL treated group.

Discussion

These results indicate that at clinically appropriate dosages, referenced by the CDC as an MIC greater than 16 ug/mL for MRSA strains, do not inhibit osteoblast cellular functions or proliferation. As shown, concentrations of vancomycin up to 500 ug/mL do not demonstrate decreased proliferation, calcium deposition, or osteoblast differentiation. Similarly, Booysen et al. explored concentrations up to 20 ug/mL,

indicating no decrease in osteoblast viability or mesenchymal stem cell differentiation (Booysen et al., 2018). Liu et al., explored concentrations beyond 1000 ug/cm^2, including 3000, 6000, and 12000 ug/cm^2 and found that continuous exposure to concentration of vancomycin greater than 1mg/cm^2 results in decreased viability 48 hours post exposure. However, they also showed that short exposure, defined as 1 hour, to concentrations of 1000 to 6000 ug/ cm^2 had no decreased cell migration or cell survivability (Liu et al 2018). Here we indicated a significantly increased dose of 5,000 ug/mL will not cause detrimental effects to the osteoblasts, when treated with only an initial dose (Figures 1, 2 and 3). Baun et al. determined that vancomycin treatment with concentrations up to 1mg/mL (1000 ug/mL) will not result in decreased cell viability or decreased ALP activity. Edin et al., also determined that concentrations up to 1000 ug/mL did not demonstrate inhibition of osteoblast replication. They did however note cell death when using concentrations of 10,000 ug/mL. As shown here, a continual dosage of 5000 ug/mL does indeed inhibit cell proliferation, calcium deposition, and ALP activity (Figure 3). Sakamoto et al., did demonstrate that vancomycin concentrations greater than 2 mM can have a detrimental effect in LLK-PK1 cells (renal tubular epithelial cells), specifically the mitochondria (Sakamoto et al., 2017). This research suggest that high doses of vancomycin may impact the mitochondria of local cells like osteoblasts. Further research is necessary to validate this finding.

It is important to note that concentrations used (50, 500, and 5000 ug/mL) are well above the mean inhibitory concentrations necessary for antimicrobial activity. As per the CDC MRSA strains with decreased susceptibility to vancomycin has a minimum

inhibitory concentration (MIC) of 4-8 ug/mL, whereas resistant strains have an MIC greater than 16 ug/mL. Therefore, both the 5 ug/mL and 50 ug/mL are both well in the range of successfully preventing resistant MRSA infections. As discussed, the pro-inflammatory state of diabetic patients results in poor fracture healing overall. In addition, diabetic patients also have an increased risk of developing infections, infections which themselves result in poor bone healing. This research aims to provide merit for an additional form of infection control in patients with increased susceptibility to both infection and poor bone healing. We have shown that single doses of up to 5,000 ug/mL do not have any detrimental effects to the periosteum derived osteoblasts.

Interestingly, this data shows that although a single dose of 5,000 ug/mL does decrease calcium deposition at day 14, by day 28 the osteoblasts recover, with calcium deposition comparable to the control (Figure 3). This could indicate that this initially high dose potentially diffuses from the site, and the cells are able to recover and function as normally. However, the importance of using lower effective ranges, with no detrimental effect to the osteoblasts cannot be understated. Further in vivo work would also need to complete to determine if local administration of vancomycin can lead to systemic effects as observed with intravenous vancomycin administration.

Conclusion

Clinically appropriate dosages of vancomycin do not affect periosteum differentiation or function. However, a continuous dose of 5000 ug/mL does inhibit cellular proliferation, calcium deposition, and alkaline phosphatase activity.

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