Concentration-dependant Response to MgO Nanoparticles

Title: Concentration-Dependent Behaviors of Bone Marrow Derived Mesenchymal Stem Cells and Infectious Bacteria toward Magnesium Oxide Nanoparticles

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Abstract

This article reports the quantitative relationship between the concentration of magnesium oxide (MgO) nanoparticles and its distinct biological activities towards mammalian cells and infectious bacteria for the first time. The effects of MgO nanoparticles on the viability of bone marrow derived mesenchymal stem cells (BMSCs) and infectious bacteria (both gram-negative *Escherichia coli* and gram-positive *Staphylococcus epidermidis*) showed a concentration-dependent behavior *in vitro*. The critical concentrations of MgO nanoparticles identified in this study provided valuable guidelines for biomaterial design toward potential clinical translation. BMSCs density increased significantly when cultured in 200 µg/mL of MgO in comparison to the Cells Only control without MgO. The density of BMSCs decreased significantly after culture in the media with 500µg/mL or more of MgO. Concentrations at or above 1000µg/mL of MgO resulted in complete BMSCs death. Quantification of colony forming units (CFU) revealed that the minimum bactericidal concentration (MBC) of MgO for *E. coli* and *S. epidermidis* was 1200µg/mL. The addition of MgO nanoparticles into the cultures increased the pH and Mg²⁺ ion concentration in the respective culture media, which might have played a role in the observed cell responses but not the main factors. *E. coli* and *S. epidermis* still proliferated significantly at alkaline pH up to 10 or with supplemental Mg²⁺ dosages up to 50, indicating bactericidal properties of MgO are beyond the effects of increased media pH and Mg²⁺ ion concentrations. MgO nanoparticles at a concentration of 200 µg/mL provided dual benefits of promoting BMSC proliferation while reducing bacterial adhesion, which should be further studied for potential medical implant applications. The use of free MgO nanoparticles yielded detrimental effects to BMSCs in concentrations above 300µg/mL. We recommend further study into MgO nanoparticle as a coating material or as a part of a composite.

Keywords:

magnesium oxide (MgO) nanoparticles, bone marrow derived mesenchymal stem cells (BMSCs), cytocompatibility, viability, bacteria, *Escherichia coli* (*E. coli*), *Staphylococcus epidermidis* (*S. epidermidis*)

1. Introduction

This article reports the quantitative relationship between the concentration of magnesium oxide (MgO) nanoparticles and its distinct biological activities towards mammalian cells and infectious bacteria for the first time. The effects of MgO nanoparticles on the viability of bone marrow derived mesenchymal stem cells (BMSCs) and infectious bacteria (both gram-negative and gram-positive) showed a concentration-dependent behavior *in vitro*. The critical concentrations of MgO nanoparticles identified in this study provided valuable guidelines for biomaterial design toward potential clinical translation.

The bioactivity and mechanical properties of MgO have recently attracted significant interest for potential biomedical applications, from bone repair to biosensing [1-5]. For bone repair, Nygren *et al.* observed that the thickness of compact bone increased by 25% after implanting MgO powder paste into bone marrow cavity of rat tibia for 3 weeks as compared with sham-operated controls. This suggested that MgO had stimulatory effects on bone healing and regeneration [6]. Clinically, MgO has also been used to improve bone mineral density when taken orally as a dietary supplement [7]. Moreover, MgO is beneficial for improving mechanical properties of poly (methyl methacrylate) (PMMA)-based bone cement. For example, MgO was incorporated into PMMA cement to enhance the fracture toughness of bone-PMMA interface [8]. Additionally, MgO powder was reported to exhibit antimicrobial properties against both gram-negative and gram-positive bacteria *in vitro*, such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) [9, 10]. These appealing properties of MgO make it a promising material for treating bone-related diseases and injuries, such as osteoporosis (a disease that decreases bone density and increases risk for bone fracture), osteomyelitis (bone infection), bone fracture, or other bone loss induced by trauma or disease.

MgO nanoparticles can further enhance the aforementioned properties considering its high surface area to volume ratio due to its nanometer size, as has been shown for other metal oxide nanoparticles [11]. Before taking advantage of their desirable properties for clinical applications, it is important to determine how MgO nanoparticles affect cellular functions, specifically the functions of relevant bone cells. BMSCs are multipotent stem cells that can differentiate into a variety of mesodermal cells, including osteoblasts (bone forming cells), and play critical roles in bone healing [12, 13]. Thus, BMSC culture provides an excellent model system for studying the cytocompatibility of MgO nanoparticles and determining clinically relevant concentration range.

The culture methods to determine material-cell interactions are not yet standardized. Previous studies have used co-seeding and sequential seeding to study the interactions between BMSCs and other cell lines [14-16]. We were interested in applying the same techniques to study the interactions between BMSCs and MgO nanoparticles. Co-seeding involves seeding the BMSCs with MgO simultaneously. Sequential seeding involves culturing BMSCs for 24 hours prior to exposure to MgO. Through these methods we aimed to model two analogous *in vivo* scenarios: the interactions between MgO nanoparticles and migrating cells (co-seeding) and the interaction between MgO nanoparticles and well-established cells within the extracellular matrix (sequential seeding).

In addition, to realize the coupled benefits of MgO nanoparticles in promoting bone growth and antimicrobial activity, it is essential to determine how MgO nanoparticles affect viability of infectious bacteria, especially the species causing orthopedic implant infection and osteomyelitis. *E. coli* is one of the most common gram-negative bacteria that cause orthopedic implant infection [17]. *Staphylococcus epidermidis* (*S. epidermidis*) is one of the most common

gram-positive infectious bacteria found in osteomyelitis [17, 18]. Thus, we are interested in evaluating the antimicrobial activity of MgO nanoparticles with both bacteria types. It is also important to include both gram-negative and gram-positive bacteria in the study to comprehend overall antimicrobial properties of a material. Minimum bactericidal concentration (MBC) is crucial for fighting clinically relevant bacterial infections and developing antimicrobial biomaterials and surfaces because MBC indicates the lowest concentration required to kill bacteria. Specifically, the MBC is the concentration that results in $\geq 90\%$ decrease in the colony forming units (CFU), with respect to the seeding density. MBC is not yet established for MgO nanoparticles. Krishnamoorthy *et al.* reported that the minimum inhibitory concentration (MIC) of MgO nanoparticles was 500 $\mu\text{g/mL}$ for *E. coli* and 1000 $\mu\text{g/mL}$ for *S. aureus* using a microtitre plate-based assay with resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one, $\text{C}_{12}\text{H}_7\text{NO}_4$) as an indicator for bacterial growth [19]. The previously reported MIC provided the basis for our study to determine the MBC of MgO nanoparticles for *E. coli* and *S. epidermidis*.

Therefore, the objective of this study was to investigate the effects of MgO nanoparticles on the viability of BMSC, *E. coli*, and *S. epidermidis*, and to determine the critical cytotoxic concentrations (CCC) for BMSCs and MBCs for bacteria. To better understand the factors that may affect the CCC and MBCs, we also analyzed the media and broth with respect to the change of pH and Mg^{2+} ion concentrations.

2. Materials and Methods

2.1 Preparation and Characterization of MgO Nanoparticles

MgO nanoparticles were procured from US Research Nanomaterials Inc. (US3310). The vendor reported that MgO nanoparticles had a 99+% purity, a diameter of 20nm, a specific

surface area of $> 60 \text{ m}^2/\text{g}$, a polyhedral morphology, a bulk density of 0.145 g/cm^3 , and a true density of 3.58 g/cm^3 . The MgO nanoparticles for BMSC and bacterial cultures were sterilized through heating at 200°C in an oven for one hour. MgO cannot be sterilized through UV irradiation because the UV affects the surface chemistry of the MgO particles by causing adsorption of O_2 and production of superoxide ions [25, 26]. MgO nanoparticles were sterilized prior to characterization so that the particles would be in the same condition during characterization as they were in *in vitro* experiments.

MgO nanoparticles were characterized before their use in cell and bacteria cultures. The morphology of MgO nanoparticles was visualized using a field emission scanning electron microscope (SEM; Philips XL30), with a secondary electron detector, at an accelerating voltage of 30 kV, a working distance of 10 mm, and an original magnification of 250,000x. MgO particle size and distribution were quantified based on SEM images using the quantitative image analysis tools in ImageJ. The morphology and crystal structure of the MgO nanoparticles were also confirmed using transmission electron microscopy (TEM; Titan Themis 300) at an accelerating voltage of 120 kV. Elemental composition of the MgO nanoparticles was confirmed using the energy dispersive X-ray spectroscopy (EDS; EDAX Leap detector attached to Philips XL30 SEM) at a spot size of 3 and an accelerating voltage of 30 kV. Crystalline phase of MgO nanoparticles was characterized using x-ray diffraction (XRD; Empyrean, PANalytical). The XRD spectrum for MgO was obtained using $\text{Cu K}\alpha$ radiation (45 kV, 40 mA) at a step size of 0.006° and dwelling time of 50 seconds using a PIXcel 1D detector (PANalytical). Phase identification was performed using the HighScore software (PANalytical).

2.2 BMSC Culture with MgO Nanoparticles and Analyses

2.2.1 Bone Marrow Derived Mesenchymal Stem Cell Culture

Rat BMSCs were extracted from the femur and tibia of juvenile Sprague Dawley rats according to the established protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California at Riverside [27, 28]. Briefly, the ends of the dissected long bones were cut using a scalpel and cell-containing bone marrow was flushed out of the marrow cavity using Dulbecco's Modified Eagle Medium (DMEM; Corning) supplemented with 10 v/v % fetal bovine serum (FBS; Hyclone) and 1 v/v % penicillin/streptomycin (P/S; Hyclone). Hereafter, DMEM with 10 v/v % FBS and 1 v/v % P/S will be referred to as DMEM. The bone marrow was collected into a sterile 15 mL tube with DMEM and large clusters were broken up using a syringe. The cell suspension was then filtered using a 70 μ m nylon strainer (Fisher Scientific, FisherbrandTM 22363548) to remove aggregates. The filtered cells were centrifuged and cultured in fresh DMEM in a T-75 flask under standard cell culture conditions (that is, a sterile, 37 °C, 5% CO₂/95% air, humidified environment). After 3 days, the media was changed to wash away non-adhered hematopoietic stem cells, leaving only the adherent mesenchymal stem cells to be cultured until they reached confluency. The pH of DMEM was adjusted to 7.4 before being used in cell culture and addition of MgO nanoparticles. BMSCs at their second passage were used for *in vitro* experiments with MgO nanoparticles.

All the experimental procedures were conducted under sterile conditions using a biosafety II laminar flow hood (Class II Type A2, Labconco) and a cell culture incubator (MCO-19AIC, Sanyo Scientific) in a certified cell culture room.

2.2.2 Co-seeding and Sequential-seeding Methods for Culturing BMSCs with MgO

Nanoparticles

Two culture methods, i.e., co-seeding and sequential-seeding, were used to study the effects of MgO nanoparticles on BMSCs *in vitro*. For the co-seeding method, MgO nanoparticles were introduced concurrently into the culture when BMSCs were seeded. Specifically, BMSCs were seeded at a density of 10,000 cells/cm² in 2 mL DMEM into each well of a 12-well tissue culture treated plate (Corning, Falcon® 353043). Immediately following, MgO nanoparticle allocations of 0.6, 0.9, 1.5, 2.1, 3.0, 3.6, 4.2, 4.8, and 6.0 mg were suspended in 1mL of DMEM and added into the respective wells. Each well had a total volume of 3mL. The resulting concentrations of MgO nanoparticles in the respective wells were 200, 300, 500, 700, 1000, 1200, 1400, 1600, and 2000 µg/mL.

For the sequential-seeding method, BMSCs were first seeded at a density of 10,000 cells/cm² into each well with 3mL of media in the 12-well tissue culture treated plates, and cultured for 24 hours to allow the BMSCs to attach. Afterwards, the DMEM was replaced with 3mL of fresh media containing the prescribed 200-2000 µg/mL of MgO nanoparticles. The BMSCs were then cultured with MgO nanoparticles at concentrations of 200- 2000 µg/mL for additional 24, 48, and 72 hours. Every 24 hours, 2.5mL of media were collected and replaced with the same volume of fresh media; the rest 0.5 mL of media were not collected in order to avoid disturbing the MgO nanoparticles at the bottom of the wells.

The BMSC cultures without MgO nanoparticles were included as the Cells Only positive control and the DMEM without cells and MgO as the Media Only control (blank reference). All the experimental and control groups were run in triplicate.

2.2.3 Post-culture BMSC Characterization and Media Analyses

After each prescribed culture period, 3mL of media was collected for media analysis and the cells were prepared for fixation and staining. Each culture well was washed with phosphate-buffered saline (PBS) to remove non-adherent or dead cells. Adherent cells were fixed using 4% paraformaldehyde (Electron Microscopy Sciences, 15714-S). The paraformaldehyde was diluted with PBS from a stock of 32% to 4 v/v % prior to fixing. The fixed cells were then stained with 4',6-diamidino-2-phenylindole dilactate (DAPI; Life Technologies) for the nuclei of the cells and Alexa Fluor[®] 488-phalloidin (Life Technologies) for F-actin. The stained cells in each well were imaged using a fluorescence microscope (Nikon Eclipse Ti-S) at 10 random locations. For each well, the number of adherent cells was counted using the analysis tools in ImageJ. BMSC adhesion density was calculated as the number of adhered cells per unit area. Each image represents a known area of the culture plate. We divided the cell count in an image by the area of the image to calculate the cell density. The average cell density calculated from all the images for one sample type was used to represent the cell density of that sample type.

The pH of the post-culture media was measured immediately after collection using a pre-calibrated pH meter (Symphony SB70P, VWR). The concentrations of Mg^{2+} and Ca^{2+} ions were measured using inductively coupled plasma optical emission spectrometry (ICP-OES; PerkinElmer Optima 8000). For the ICP-OES analysis, the collected post-culture media was first diluted to 1:100 in deionized (DI) water, and then fed into the instrument for the measurement using an auto sampler. Concentrations of Mg^{2+} and Ca^{2+} ions were calculated based on the calibration curves generated using Mg and Ca standards (Perkin Elmer) diluted to the ranges of 0.5–5.0 and 0.1–1.0 mg/L, respectively.

2.2.4 Behavior of MgO Nanoparticles in DMEM without BMSCs

MgO nanoparticles were cultured in DMEM in acellular conditions to determine their effects on the media. Specifically, MgO nanoparticles at the prescribed 200-2000 µg/mL concentration were incubated in DMEM without cells under standard cell culture conditions for 24 hours. Subsequently, the media were collected for pH measurements and analyses of Mg²⁺ and Ca²⁺ concentrations using ICP-OES, as described earlier.

Considering the buffering effects of DMEM under standard cell culture conditions, it is necessary to monitor the media pH more frequently within the first 24 hours of incubation. To evaluate the effect of MgO nanoparticles on pH, concentrations of 0, 200, and 1000 µg/mL of MgO nanoparticles in 3mL of DMEM were selected. The media pH was measured after incubation for 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 24 hours. The pH at 0 minute was the media pH measured immediately after adding MgO nanoparticles but before placing into the cell culture incubator.

2.3 Bacterial Culture with MgO Nanoparticles and Analyses

2.3.1 Bacterial Culture

E. coli (ATCC[®] 25922) and *S. epidermidis* (ATCC[®] 35984) were cultured in Luria-Bertani Broth (LBB; Sigma Aldrich, L3022) and Tryptic Soy Broth (TSB; Sigma Aldrich, 22092), respectively. Frozen stocks of each bacterial type were stored at -80°C. A sterile loop was used to transfer a portion of the frozen stocks to their respective broths. The bacteria were cultured in a shaker incubator (Incu-shaker Mini, Benchmark Scientific) at 37°C and 250 rpm for 16 hours. For *S. epidermidis*, an aliquot of 100 µL from the culture grown overnight was added to 5 ml of fresh broth and cultured for another 4-6 hours. Afterwards, the bacteria were counted using a hemocytometer (Hausser Bright-Line 3200, Hausser Scientific) and diluted to a

concentration of 5×10^5 cells/mL in LBB or TSB because this is the clinically relevant concentration for orthopedic infections [29]. Subsequently, 3 mL of *E. coli* in LBB or *S. epidermidis* in TSB were added to each well of a 12 well non-tissue culture treated polystyrene plate (Corning, Falcon® 351143), and MgO nanoparticles with the prescribed mass of 0.6, 0.9, 1.5, 2.1, 3.0, 3.6, 4.2, 4.8, and 6.0 mg were added immediately after. As a result, *E. coli* and *S. epidermidis* were cultured with 200-2000 µg/mL of MgO nanoparticles, and incubated for 24 hours in a shaker incubator at 37°C and 120rpm. The co-seeding of the bacteria and MgO mimics the *in vivo* scenario in which bacteria are introduced with medical devices or implants. The shaking speed for the culture plates was reduced from the initial speed of 250 rpm to 120 rpm to prevent spillage between wells. After 24 hours, the respective bacteria in the LBB or TSB were collected for analyses. The *Bacteria Only* group without MgO nanoparticles and the *Broth Only* group without MgO nanoparticles and bacteria were included as the positive control and blank reference, respectively.

2.3.2 Post-culture Bacterial Viability and Broth Analyses

After the respective bacteria were cultured with MgO nanoparticles, the bacterial concentrations in the collected broth were analyzed using two different methods, i.e., measuring optical absorbance using a microplate reader (Infinite M200 Pro, Tecan) and measuring the colony forming units (CFU) plated on agar plates. Viability of the suspended *E. coli* and *S. epidermidis* was determined through evaluation of colony forming units (CFU) on Luria Bertani (LB) and Tryptic Soy (TS) Agar (Sigma Aldrich) plates, respectively. The *E. coli* in the LBB was serially diluted to $1:10^6$ for the control group and the experimental groups cultured with 200-1200 µg/mL MgO nanoparticles, while the groups with 1400-2000 µg/mL MgO nanoparticles were not diluted. The *S. epidermidis* in the TSB was serially diluted to $1:10^6$ for the control group

and the experimental groups cultured with 200 or 300 $\mu\text{g/mL}$ MgO nanoparticles, $1:10^4$ for the groups with 500 $\mu\text{g/mL}$ MgO nanoparticles, $1:10^2$ for the groups with 700 $\mu\text{g/mL}$ MgO nanoparticles, and $1:10^{-1}$ for the groups with 1000-2000 $\mu\text{g/mL}$ MgO nanoparticles. The dilution factor was selected to give a reasonably countable number of CFUs. Each bacterial suspension was diluted with its respective broth and 100 μL of the diluted suspension was pipetted onto the respective agar plates. The bacterial suspension was then evenly spread across the respective agar plates. The agar plates were inverted so that the agar was on the top side, and then incubated for 16-18 hours. The number of colonies formed on the respective agar plates was counted, and multiplied by the dilution factor to obtain the CFU/ml values.

The pH of the post-culture LBB and TSB was measured using a pre-calibrated pH meter (Symphony SB70P, VWR). The post-culture LBB and TSB was diluted to 1:50 with DI water and analyzed using ICP-OES to determine the Mg^{2+} and Ca^{2+} ion concentrations in each broth. All the experimental and control groups were run in triplicate.

2.3.3 Characterization of Bacterial Adhesion and Morphology by SEM

To characterize the bacterial adhesion and morphology after they were cultured with MgO nanoparticles, *E. coli* and *S. epidermidis* was cultured with 200 to 2000 $\mu\text{g/mL}$ MgO nanoparticles and prepared for SEM imaging. Before placing the bacteria or MgO into each culture well, a sterilized 1cm^2 square borosilicate glass slide (Fisher Scientific, Fisherbrand™ cover glass 12-542-B) was placed into the bottom of each well. The glass slides were previously cut to 1 cm x 1 cm squares, using a diamond saw (DynaCut 1500HD). The bacterial culture method was similar as described above. After the respective bacteria were cultured with the prescribed concentrations of MgO nanoparticles on the glass slides for 24 hours, each well was rinsed with Tris-buffered saline (Sigma Aldrich) to remove the bacteria that did not attach. The

bacteria were then fixed in 10% glutaraldehyde for 1 hour. The 10% glutaraldehyde solution was previously diluted from a 25% glutaraldehyde solution (Sigma-Aldrich) in a Tris-buffered saline. Each sample was then rinsed 3 times with a Tris-buffered saline, and dried in air at room temperature for at least 24 hours. These dried glass slides with adherent bacteria were then coated with Pt/Pd using a sputter coater (108 Auto Sputter Coater, Cressington Scientific.) at 20mA for 60 seconds, in preparation for scanning electron microscopy (SEM; Philips XL30). Representative images were taken using a secondary electron detector, at an accelerating voltage of 10 kV, a working distance of 10 mm, and an original magnification of 10,000x. The bacterial culture with glass and subsequent fixation procedure were intended for SEM imaging of bacterial adhesion and morphology, and were not used for bacterial quantification.

2.3.4 The Effects of MgO Nanoparticles on LBB and TSB without Bacteria

MgO nanoparticles ranging from 200 to 2000 µg/mL were incubated in LBB or TSB in acellular conditions for 24 hours to determine their specific effects on the pH of broth and the ionic concentrations in broth. At the end of 24 hours, the broth was collected for pH measurements and analyses of Mg^{2+} and Ca^{2+} concentrations using ICP-OES, similarly as described earlier.

2.3.5 Bacterial Culture with Increasing pH and Mg^{2+} ion Concentrations

To determine whether the ionic byproducts of MgO nanoparticles (OH^- and Mg^{2+}) contributed to its antimicrobial properties, we cultured *E. coli* and *S. epidermidis* in the respective broth with initial pH adjusted to 7, 7.5, 8, 8.5, 9, 9.5, and 10 or in the respective broth supplemented with 1, 5, 10, 15, 20, 30, 40, and 50 mM of Mg^{2+} ions. For the pH study, the broth pH was intentionally adjusted to 7-10 using sodium hydroxide (NaOH, Strem Chemicals). For the Mg^{2+} ion dosage study, Mg^{2+} ions were added into the respective broth using a stock solution

of magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, Sigma Aldrich). The stock solution was prepared by dissolving 1.525g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 50mL of LBB or TSB respectively, to achieve the Mg^{2+} ion concentration of 150mM. The 150mM stock solution was diluted with the respective broth to obtain the doping solutions with Mg^{2+} ion concentrations from 1 to 50mM. The doping solutions were added into the respective bacterial culture to give the supplemental Mg^{2+} ion dosages of 1-50 mM. To clarify, the values reported as supplemental Mg^{2+} dosages do not include the Mg^{2+} ions already present in the broth. It is important to note that 0mM does not actually mean there were no Mg^{2+} ions present in the broth, but rather that no additional Mg^{2+} ions were added into the broth to serve as a control group.

After 24 hours of culture, the bacteria and respective broth were analyzed to determine the specific effects of each soluble factor on the total bacterial concentration and bacteria viability, as well as the changes in broth pH and ionic concentrations, using the same method as described earlier.

2.4 Statistical Analysis

All experiments were run in triplicate. All data sets were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey highest significant difference post hoc test. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Characterization of MgO Nanoparticles

SEM images of MgO were taken immediately after sterilization and before BMSC and bacterial culture (Figure 1A). The TEM image of MgO revealed that the particles mostly have near spherical morphology and crystalline structure (Figure 1B), in agreement with the vendor's

characterization [30]. EDS results confirmed the atomic composition was close to MgO (Figure 1C). The average particle diameter for MgO was 23 ± 5 nm (Figure 1D). The standard peaks for MgO were observed in the XRD spectrum, indicating that the expected crystalline form of MgO was present (Figure 1E). Additional peaks in the XRD pattern of the MgO sample also indicated the presence of $\text{Mg}(\text{OH})_2$ phase. MgO is hygroscopic and can readily react with water in the atmosphere to form $\text{Mg}(\text{OH})_2$, which is most likely the reason for the presence of $\text{Mg}(\text{OH})_2$ peaks in the XRD spectrum.

3.2. BMSC Morphology, Adhesion Density and Respective Media Analyses

3.2.1 BMSC Adhesion Density and Media Analyses with Co-seeding

Based on the prior research, the 0 to 2000 $\mu\text{g/mL}$ concentration range of MgO nanoparticles was studied to determine the CCC for BMSCs [19]. Morphology and density of BMSCs that were cultured with MgO nanoparticles through the co-seeding method was characterized using fluorescence microscopy (Figure 2). The BMSC viability significantly reduced after 24 hours of incubation with 200 $\mu\text{g/mL}$ of MgO nanoparticles and near to complete cell death at or above 300 $\mu\text{g/mL}$, when compared with the positive control (Cells Only), as quantified in Figure 2D. Surviving cells exhibited abnormal morphology after co-seeding with 200 to 300 $\mu\text{g/mL}$ of MgO nanoparticles, appearing smaller and exhibiting signs of possible membrane damage, as highlighted in Figure 2 A-B.

For the co-seeding method, the trends of media analyses were highlighted using linear regression, as shown in Figure 3. The average pH of the culture media showed an increasing trend with increasing concentrations of MgO nanoparticles (Figure 3A). The average pH of the culture media with 200 and 300 $\mu\text{g/mL}$ MgO nanoparticles were higher than that of the *Cells Only* control, but not statistically significant; the pH for the rest of experimental groups was

significantly higher than the Cells Only control. Mg^{2+} concentration showed a similar increasing trend with increasing concentrations of MgO nanoparticles (Figure 3B). The average Mg^{2+} concentration in the culture media with 200 $\mu\text{g/mL}$ MgO nanoparticles were higher than that of the *Cells Only* control, but not statistically significant; Mg^{2+} concentration for the rest of experimental groups was significantly higher than the Cells Only control. In contrast, Ca^{2+} concentration showed a decreasing trend with increasing concentrations of MgO nanoparticles (Figure 3C). Ca^{2+} concentration for all experimental groups was statistically lower than that of the *Cells Only* control.

3.2.2 BMSC Adhesion Density and Media Analyses with Sequential-Seeding

The morphology of BMSCs that were cultured with MgO nanoparticles through the sequential-seeding method was observed using fluorescence microscopy (Figure 4). Qualitatively, cells cultured with 200 $\mu\text{g/mL}$ MgO exhibited healthy morphology and grew to confluency (Figure 4A-A"), similar to the Cells Only control group (Figure 4E-E"). At or above 300 $\mu\text{g/mL}$, the cells appeared smaller and some showed signs of membrane damage, similar to the experimental groups under the co-seeding method. The sequential-seeding method closely represents how cells in the body would react to the use of MgO nanoparticles, since BMSCs in our body would be well established in their extracellular matrix before the introduction of implant materials such as MgO.

The BMSC adhesion density was quantified based on the fluorescence images of sequentially seeded cultures (Figure 5). The cell density significantly increased after the cells were incubated with 200 $\mu\text{g/mL}$ of MgO nanoparticles for 72 hours, when compared with all the other conditions. All samples sequentially-seeded with 300 $\mu\text{g/mL}$ or more of MgO showed significant decrease in cell density during the first 24 hours. At 300 $\mu\text{g/mL}$, no statistical

difference was detected at 48 and 72 hours when compared with the *Cells Only* control. This indicated that the cells were able to recover from MgO exposure at 300 µg/mL. The cells cultured with MgO concentrations at or above 500 µg/mL did not recover during 48 and 72 hours of culture; the cell density was significantly lower than the Cells Only control at the respective time points and the experimental groups of 200 and 300 µg/mL at 72 hours.

For the sequential-seeding method, the trends for media pH and Mg^{2+} concentration in the media were highlighted using linear regression, as shown in Figure 6. During the first 24 hours, the pH of the cell culture media increased as the concentration of MgO increased (Figure 6A), similar to the co-seeding method. At 48 and 72 hours, the pH increase was smaller when comparing each condition with their respective pH at 24 hours. The pH increased to the maximum of 8.7 at the highest MgO concentration being studied, i.e., 2000 µg/mL. The trend of increasing Mg^{2+} concentration with increasing MgO concentration during the first 24 hours of sequential culture (Figure 6B) was similar to the co-seeding method. At 48 and 72 hours, Mg^{2+} concentration continued to show an increasing trend with increasing MgO concentration, but the extent of increase was much smaller. The Ca^{2+} concentration showed a decreasing trend during the first 24 hours of sequential culture as MgO concentration increased, but this trend did not continue at 48 and 72 hours (Figure 6C). The trends in pH, Mg^{2+} and Ca^{2+} concentrations indicated that the dissolution of MgO or its effect on media occurred mostly within the first 24 hours of culture.

3.2.3 The Effects of MgO Nanoparticles on DMEM in Acellular Conditions

The addition of MgO into DMEM showed similar effects on the media pH, Mg^{2+} concentration, and Ca^{2+} concentration under acellular conditions (Figure 7). As MgO concentration increased, the media pH and Mg^{2+} concentration showed increasing trends. There

were no statistically significant differences in pH or Mg^{2+} concentration between 200-300 $\mu\text{g/mL}$ and the Media Only control. At and above 500 $\mu\text{g/mL}$, both pH and Mg^{2+} concentration were significantly higher than the Media Only control. Conversely, the Ca^{2+} concentration showed a decreasing trend as MgO concentration increased (Figure 7C). There were no statistically significant differences in Ca^{2+} concentration in media cultured with 200-300 $\mu\text{g/mL}$ MgO as compared with the Media Only control. At and above 500 $\mu\text{g/mL}$, there was statistically significant decrease of Ca^{2+} in the media with respect to the Media Only control.

After the addition of MgO nanoparticles of 200 or 1000 $\mu\text{g/mL}$ into the media under acellular conditions, we observed that the average media pH reached its peak at 15 minutes and then decreased over the 24 hours of culture (Figure 8). Specifically, at 15 minutes, the mean pH of media with 200 $\mu\text{g/mL}$ MgO was 8.57, and the pH of the media with 1000 $\mu\text{g/mL}$ MgO was 9.37. At the end of 24 hours, the pH for media with 200 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$ of MgO decreased to 7.78 and 8.01, respectively.

3.3. Bacteria Adhesion, Viability, and Respective Broth Analyses

3.3.1 Bacteria Adhesion and Viability in Exposure to MgO Nanoparticles

E. coli and *S. epidermidis* adhered on the glass slides were visualized using SEM, after culture with MgO nanoparticles at concentrations of 200 - 2000 $\mu\text{g/mL}$ (Figure 9). As compared with the Bacteria Only control, *E. coli* appeared to have a normal morphology, but less bacteria and smaller clusters were present when cultured with increasing concentrations of MgO nanoparticles from 200 to 700 $\mu\text{g/mL}$ (Figure 9 A⁻-D⁻). MgO nanoparticles at concentrations of 1000 - 2000 $\mu\text{g/mL}$ caused complete bacteria death and no bacteria were found on the glass slides (Image not shown). The *S. epidermidis* showed similar trends as *E. coli* after culture with MgO nanoparticles (Figure 9 A⁺-C⁺). As compared with the Bacteria Only control, *S.*

epidermidis appeared to have a normal morphology, but far less bacteria and fewer clusters were observed as MgO concentrations increased. Qualitatively, the presence of MgO nanoparticles resulted in reduction of adherence for both gram-negative and gram-positive bacteria, beneficial for preventing biofilm formation.

Bacterial viability was quantified through CFU (Figure 10). We also quantified live and dead bacteria concentration through optical absorbance measurements, which further confirmed our CFU measurements (Data not shown). The absorbance measurements quantify all bacteria in broth and do not distinguish live or dead bacteria, while CFUs measured from agar plating only quantify viable bacteria. The presence of MgO nanoparticles did not affect the absorbance reading. Based on CFU quantification, *E. coli* viability decreased significantly when cultured with MgO nanoparticles at and above 1200µg/mL, indicating MBC for *E. coli* was 1200 µg/mL (Figure 10). *S. epidermidis* viability decreased when cultured with MgO nanoparticles at 500 and 700 µg/mL as compared with the Bacteria Only control, making these concentrations bacteriostatic (Figure 10). *S. epidermidis* viability decreased significantly when cultured with MgO nanoparticles at and above 1200µg/mL, indicating MBC for *S. epidermidis* was 1200 µg/mL. At MgO concentrations of 200-300 µg/mL, the CFUs of *E. coli* and *S. epidermidis* were significantly greater than their respective controls, indicating that low concentration of MgO might enhance bacterial growth.

Collectively, our results suggested that MgO nanoparticles at certain concentrations not only reduced bacterial viability in broth, but also reduced bacterial adhesion density onto glass surfaces. Inhibiting bacterial adhesion onto an implant surface is important to reduce implant-associated infections, because adhered bacteria are much harder to treat and systemic administration of antibiotics is often ineffective [31].

The pH of the LBB for *E. coli* culture and TSB for *S. epidermidis* culture was plotted in Figure 11A. The pH of the *E. coli* culture with 200 to 1000 µg/mL of MgO all showed that average pH increased to 8.8 or greater, significantly greater than the Broth Only controls. When compared with the Bacteria Only control, the pH of the *S. epidermidis* culture did not change much when cultured with 200 to 300 µg/mL of MgO, and started to increase steadily as the MgO concentration increased to and above 500 µg/mL. When comparing the pH of the MgO-free LBB controls with and without *E. coli*, it appeared that *E. coli* increased pH to 8.7, which is alkaline. When comparing the pH of the MgO-free TSB controls with and without *S. epidermidis*, it appeared that the *S. epidermidis* reduced pH to 5.7, which is acidic.

Both the LBB for *E. coli* culture and TSB for *S. epidermidis* culture showed increasing Mg^{2+} and Ca^{2+} concentrations with increasing MgO (Figure 11 B,C). At 2000 µg/mL of MgO, Mg^{2+} concentration reached 42 mM for *E. coli* culture and 36mM for *S. epidermidis* culture, respectively.

3.3.2 The Effects of MgO Nanoparticles on LBB and TSB in Acellular Conditions

In acellular conditions, the pH and Mg^{2+} concentration showed increasing trends for both LBB and TSB as MgO concentration increased (Figure 12). The average pH of the Broth Only controls was 7.1 for LBB and 8.1 for TSB. Each group with MgO resulted in higher average pH when compared with the Broth Only control. Statistically significant increased pH was found in all groups of LBB with MgO. The pH of TSB groups with MgO did not show statistically significant difference when compared with the Broth Only control until MgO concentration increased to 1600 µg/mL due to the large deviation in the Broth Only control. All groups with MgO showed statistically higher Mg^{2+} concentration than the Broth Only controls. Mg^{2+} concentration steadily increased with increasing MgO concentrations. In the groups with MgO,

Ca^{2+} concentration generally decreased with increasing MgO for both broth types. For LBB, all groups with MgO had significantly less Ca^{2+} compared to the Broth Only control. In the case of TSB, we observed greater average Ca^{2+} concentration in the groups with 200-500 $\mu\text{g/mL}$ of MgO than the Broth Only control, but only statistically significant at 200 and 500 $\mu\text{g/mL}$. At and above 700 $\mu\text{g/mL}$, the average Ca^{2+} concentration in TSB was lower than the Broth Only control, but not statistically significant.

3.3.3 The Effects of Mg^{2+} Ion Dosing on Bacteria Viability and Growth

Bacterial culture with supplemental Mg^{2+} ions did not lead to bactericidal or bacteriostatic effects on *E. coli* or *S. epidermidis* because all of the evaluated groups with supplemental Mg^{2+} dosages resulted in significant bacterial growth since seeding, similar to the 0mM dosage group (Figure 13). CFU quantification showed similar results with significant bacterial growth for the groups with supplemental Mg^{2+} dosages, similar to the 0mM group (no supplemental dosage). The groups supplemented with 10, 15, 40, and 50mM Mg^{2+} ions showed statistically significant increase in viable *S. epidermidis* (Figure 13).

The pH of the broths with supplemental Mg^{2+} dosages remained relatively unchanged with only a slight decrease in pH at dosages of 20mM or higher for both *E. coli* and *S. epidermidis* (Figure 14A). With increasing Mg^{2+} concentration, we observed the expected increase in final Mg^{2+} concentration for both LBB and TSB (Figure 14 B). Ca^{2+} concentration of the LBB with *E. coli* increased with increasing supplemental Mg^{2+} dosage but had a sharp decrease at 50mM (Figure 14 C). For TSB with *S. epidermidis*, Ca^{2+} concentration increased with increasing supplemental Mg^{2+} dosage.

3.3.4 The Effects of Initial pH on Bacteria Viability and Growth

Increasing initial broth pH up to 10 did not lead to bactericidal or bacteriostatic effects on *E. coli* or *S. epidermidis* because all of the evaluated groups with increased pH resulted in significant bacterial growth since seeding, similar to the control group with initial pH of 7 (Figure 15). CFU quantification showed statistically significant but slight decrease in *E. coli* CFUs at pH of 10; some *S. epidermidis* groups with initial pH adjusted to 7.5, 8.5, 9, 9.5, and 10, showed statistically greater CFUs than the control group with initial pH of 7.

The final pH of LBB for *E. coli* culture changed to a narrow range of 8.7 - 8.8 and the final pH of TSB for *S. epidermidis* culture decreased to 5.5 - 8.2, even though their initial pH values were adjusted to 7-10, respectively (Figure 16A). Adjusting the initial broth pH to higher values led to decreasing trends in Mg^{2+} concentrations, with some statistical significance at pH 7.5, 8.5 and above for *E. coli* and at pH 9 and above for *S. epidermidis* (Figure 16B). Ca^{2+} concentration decreased when the initial broth pH values were adjusted to 7.5 - 10 as compared with the control group at initial pH 7, for both types of bacteria (Figure 16C).

4. Discussion

4.1 BMSC Viability

The effects of MgO nanoparticles on BMSC adhesion density and viability depend on MgO concentration and *in vitro* culture methods. With co-seeding method, BMSC adhesion density all decreased when cultured with MgO, as compared with the Cells Only control, because BMSCs were likely to have more direct-contact with MgO nanoparticles during co-seeding when the cells were not yet adhered to the substrates and not yet established in culture, which might have made the BMSCs more vulnerable to MgO nanoparticles. MgO nanoparticles were also

reported to increase bacterial death with more direct-contact via shaking [9], which suggested that direct-contact with MgO might be a key factor causing cell death. In contrast, with sequential-seeding method, BMSC density was significantly greater than the Cells Only control at 72 hours when cultured with 200 $\mu\text{g/mL}$ of MgO. Moreover, the BMSCs with sequentially seeded 300 $\mu\text{g/mL}$ of MgO showed similar cell density as the Cells Only control at 72 hours. These results suggested that BMSCs were less vulnerable to the effects of MgO under the sequential-seeding method, because they adhered to the culture plates and established themselves in the culture before exposure to MgO. Thus, MgO nanoparticles, in small dosages, such as 200 $\mu\text{g/mL}$, are beneficial for bone regeneration, because they could enhance proliferation of the BMSCs. The sequential-seeding method represents how the cells would interact with newly implanted MgO more closely than the co-seeding method, and, thus, will be the focus of the following discussion. For the purposes of this study, we define CCC as the concentration of the material of interest, in our case of MgO, which results in 50+% cell deaths with respect to the seeding density. Our results suggested that the CCC for 20nm MgO nanoparticles to BMSCs were between 300 and 500 $\mu\text{g/mL}$ under the sequential-seeding method. Figure 17 summarizes the critical MgO concentrations and their effects on BMSCs under the sequential-seeding method.

MgO nanoparticles resulted in increase of media pH; intuitively, the pH increase might be a factor contributed to the observed BMSC death at or above 500 $\mu\text{g/mL}$ of MgO. The monitoring of pH change over a 24-hour period showed that the pH increase occurred rapidly within the first 15 min and then decreased (Figure 8). The peak pH of media with 200 $\mu\text{g/mL}$ of MgO was 8.57 and the peak pH of media with 1000 $\mu\text{g/mL}$ of MgO was 9.37. The reduction in the pH values at 48 and 72 hours in the sequentially seeded culture (Figure 6A), indicated that

most of the MgO nanoparticles dissociated in the first 24 hours and thus released more OH^- during that initial 24 hours. Previous work from our lab has shown that there was no statistical difference in BMSC density when cultured in DMEM with initial pH values adjusted up to 9.0 [28]. It is possible that pH was a factor contributing to the cell death observed at and above 1000 $\mu\text{g/mL}$ of MgO, considering the peak pH reached 9.37, above 9.0. However, the fact that the BMSC death was observed at 200 $\mu\text{g/mL}$ of MgO in the co-seeded condition but not in the sequentially seeded condition indicated that there might be other reasons for the cytotoxic effects of MgO nanoparticles at these low concentrations of MgO where the peak pH was below 9.0.

Our results are in agreement with the theoretical dissolution of MgO, which resulted in increase in pH and release of Mg^{2+} ions [20-23]. When exposed to water, MgO absorbs water to form $\text{Mg}(\text{OH})_2$ due to its hygroscopic nature [20]. MgO is only slightly soluble in water, with a solubility of 0.086 g/L at 30 °C, which is much greater than the solubility of 0.012 g/L for $\text{Mg}(\text{OH})_2$. Despite the low solubility, MgO can dissociate in water by the overall reaction: $\text{MgO} + \text{H}_2\text{O} \rightarrow \text{Mg}^{2+} + 2\text{OH}^-$ [21]. $\text{Mg}(\text{OH})_2$ molecules dissolved in water also dissociate and release Mg^{2+} ions and hydroxide anions (OH^-) [22]. It is important to mention that the actual chemical interactions of MgO with physiological fluids are very complex, due to the presence of calcium, phosphate, carbon dioxide, and other ions and proteins. MgO and $\text{Mg}(\text{OH})_2$ also form on the surface of Mg-base metallic alloys when exposed to aqueous physiological solutions, resulting in the release of OH^- and Mg^{2+} ions [23, 24].

It is clear that the cells do have an effect on the Mg^{2+} concentrations. Specifically, the average Mg^{2+} concentrations in media with 2000 $\mu\text{g/mL}$ of MgO were similar for both co-seeding and sequential-seeding conditions (44 mM), which were greater than that for acellular conditions at 24 hours (34 mM). It is speculated that either the BMSCs promoted MgO

dissociation or their death led to the release of intracellular Mg^{2+} ions; as a result, Mg^{2+} concentrations increased as compared with the acellular conditions. Intracellular magnesium concentrations range from 5 to 20 mmol/L [32, 33]. The release of right amount of Mg^{2+} ions at the right moment might have contributed to the enhanced proliferation at 200 $\mu\text{g/mL}$ of MgO in the sequentially-seeded culture. Figure 18A plots the correlation between Mg^{2+} concentrations and BMSC density at 24 hours for both co-seeding and sequential-seeding conditions. As Mg^{2+} concentrations increased with increasing MgO nanoparticles, a drop in cell density occurred. However, it is unlikely that Mg^{2+} ions caused BMSC death, since a recent study showed no adverse effects with supplemental Mg^{2+} ions up to 27mM [34]. Another recent study reported that Mg^{2+} ions enhanced osteogenic activity of BMSCs [35]. The release of right amount of Mg^{2+} ions at the right time through MgO dissociation could be beneficial for promoting bone regeneration.

The increase in Mg^{2+} concentrations with increasing MgO correlated with a decrease in Ca^{2+} concentration; this trend was observed in 24 hours of co-seeding, sequential-seeding, and acellular culture conditions. The decrease in Ca^{2+} ions with increasing MgO concentrations might indicate that the release of Mg^{2+} ions promoted the precipitation of Ca^{2+} ions. It has been reported that the incorporation of MgO into bioactive glasses resulted in formation of thicker apatite layers [36], most likely due to the increased precipitation of Ca^{2+} ions that promoted apatite layer formation. If the increase in Mg^{2+} ions caused the precipitation of calcium-containing salts during the first 24 hours, it is possible that the precipitated salts were rinsed away during the media exchange at the end of the 24-hour and 48-hour time points in the sequential-seeding method. This would explain why the Ca^{2+} concentration remained the same as the control for the samples with MgO at 48 and 72 hours. The Ca^{2+} concentrations at the MgO

concentrations of 200-2000 $\mu\text{g/mL}$ were different in the sequential-seeded culture versus acellular condition; the presence of BMSCs increased the Ca^{2+} concentrations at low MgO concentrations when they were viable and decreased the Ca^{2+} concentrations at high MgO concentrations when they were dead. The intracellular concentration of calcium ions is 10,000 times lower than that in the plasma, i.e., $<0.0002 \text{ mmol/L}$ intracellularly, compared with $\sim 2 \text{ mmol/L}$ in the plasma [37]. Thus, the release of intracellular Ca^{2+} is negligible, and it is more likely that higher MgO concentrations promoted Ca^{2+} deposition, which resulted in lower Ca^{2+} concentrations in the media.

In order to take full advantage of the benefits provided by MgO nanoparticles, it may be necessary to use MgO in a composite or as a coating, rather than using it alone by itself. The CCC for 20nm MgO nanoparticles was determined to be between 300 and 500 $\mu\text{g/mL}$. MgO nanoparticles in the 200 $\mu\text{g/mL}$ dosage in the sequentially seeded culture were found to enhance BMSC proliferation. This may be due to the release of Mg^{2+} ions from MgO dissociation, and Mg^{2+} ions were known to enhance osteogenic activity of BMSCs [35]. The composites incorporating MgO nanoparticles can potentially utilize the benefits of MgO, while mitigating the toxicity. For example, a recent *in vitro* study showed that MgO nanoparticles enhanced bone cell adhesion and proliferation when MgO was incorporated into a polymer to form a composite [38]. *In vivo* studies also indicated that MgO-containing pastes and scaffolds led to enhanced bone formation [5, 6, 39]. Additionally, using MgO nanoparticle as a coating material could also decrease bacterial adhesion, thereby reducing biofilm formation and infection.

4.2 Bacteria Viability

Clearly, MgO nanoparticles at the concentrations at and above 1200 $\mu\text{g/mL}$ are bactericidal to both *E. coli* and *S. epidermidis*. That is, MBC of MgO nanoparticles for *S.*

epidermidis and *E. coli* are 1200 µg/mL, which is well above the tolerable levels for BMSCs.

This suggests that bare MgO nanoparticles may not be optimal to be used directly as an antimicrobial agent in solutions or body fluids. It is important to emphasize that MBC defines the bactericidal effect to the bacteria suspended in the fluid, and MgO nanoparticles reduced the adhesion and cluster size of both *E. coli* and *S. epidermidis* when the concentration was as low as 200µg/mL (Figure 9). Bacterial clusters are far more likely to develop a biofilm than individual bacterial cell, and bacterial colonies in biofilm are more difficult to treat. Reduction in bacterial cluster number and size is beneficial because it decreases the risk of bacterial biofilm formation. Collectively, the right amount of MgO nanoparticles, e.g., 200µg/mL, could provide dual benefits, i.e., promoting BMSC proliferation while reducing bacterial adhesion and infections.

A previous study showed that MIC of MgO nanoparticles for *E. coli* was 500µg/mL based on a microtitre plate-based assay [19]. However, our CFU quantification suggested that there were no statistically significant decrease in *E. coli* viability in broth at 500µg/mL of MgO. This might be due to the difference between the microtitre plate-based assay and CFU quantification from agar plating.

The exact mechanisms for the antimicrobial properties of MgO nanoparticles are not yet confirmed. Since MgO nanoparticles caused increase in pH and Mg^{2+} concentration, we examined whether pH or Mg^{2+} concentration are the main factors contributing to bacterial death. It turns out the increase in pH and Mg^{2+} concentration did not reduce bacterial viability. The pH of LBB and TSB increased more with respect to their controls with increasing MgO concentration, than the pH of the DMEM. This is likely because the buffering effect of LBB and TSB in the bacterial culture environment is not as good as DMEM in the BMSC culture environment. Both *E. coli* and *S. epidermidis* proliferated significantly when cultured in LBB

and TSB, respectively, up to a pH of 10. The pH at the end of 24 hours showed that both *E. coli* and *S. epidermidis* could adjust the pH of their environment (Figure 16). *E. coli* tended to adjust pH to 8.6 to 8.8 no matter what initial pH was, and *S. epidermidis* adjusted pH to 5.5 when the initial pH was 7-8, and gradually increased to 8.2 when the initial pH increased to 10. LBB for *E. coli* culture contains no buffering agents. TSB contains dipotassium hydrogen phosphate at a concentration of 14mM as a buffering agent. The pKa of hydrogen phosphate buffer is 7.21. *S. epidermidis* produced acidic waste products that could mediate pH increase induced by MgO dissociation. The ability of these bacteria to adjust their environment makes them resilient to pH effects. The viability of both types of bacteria showed initial increase followed by a sharp decrease with increasing Mg^{2+} concentration from increasing MgO concentration (Figure 18 B, C). However, the release of Mg^{2+} from MgO, is unlikely to be the source of antimicrobial properties of MgO nanoparticles, because both *E. coli* and *S. epidermidis* proliferated significantly when cultured with supplemental Mg^{2+} dosage up to 50mM.

When *E. coli* and *S. epidermidis* were cultured with MgO, both LBB and TSB showed an increase in Ca^{2+} concentration over the native amount in each broth. In gram negative bacteria, such as *E. coli*, the polysaccharides that make up most of the biofilms are either neutral or polyanionic [31]. Presence of calcium and magnesium cations could enhance biofilm formation for such bacteria because these cations could cross link with polymer strands, thus strengthening binding force. However, we found that the increase of calcium ion concentration did not enhance biofilm formation by *E. coli*. This further showed effectiveness of MgO against bacterial infection. Even though there were more cations present in the broth with increasing MgO concentration, these cations did not increase bacterial adhesion or biofilm formation. The SEM images showed fewer adhered bacteria as MgO concentration increased. For gram positive

bacteria, including staphylococci, the polysaccharides are cationic [31]. The presence of cations would have no enhancing effect on the formation of *S. epidermidis* biofilm.

CFU quantification in a prior study showed lower *E. coli* viability when the bacteria were cultured with magnesium-yttrium alloy that had oxide layers on the surface than that lacked initial oxide layers [40], which suggested oxide layers on the surface played an important role on the antimicrobial activity of magnesium and its alloys.

One proposed mechanism for the antimicrobial activities of MgO suggested that the interaction of MgO and bacterial cell wall/membrane caused damage to the cell wall/membrane, leading to leakage of minerals, proteins, and genetic material resulting in cell death [41]. Previous studies also indicated that metal oxide nanoparticles could spontaneously produce reactive oxygen species (ROS) [9, 42, 43]. MgO, in particular, has been shown to enhance lipid peroxidation [19]. It is believed that superoxides, hydrogen peroxide, and/or hydroxyl radicals may be produced from consecutive oxidation-reduction reactions occurring at the MgO surface. Superoxide is also stable under alkaline conditions and has higher diffusion distance with increasing pH [44]. Superoxide from MgO can interact with the carbonyl group in the peptide and cause degradation of proteins [45]. If this were the case, ROS would be responsible for the antimicrobial properties of MgO. A recent study, however, suggested that MgO exhibited antimicrobial activity against *E. coli*, in the absence of ROS, through membrane damage [46]. According to Leung *et al.*, there was no expression of ROS-related proteins, which indicated ROS production was not part of the bactericidal properties of MgO. This study measured the protein levels in the first 20 minutes of exposure to MgO. Longer studies in the absence of ROS may be necessary to confirm this mechanism. We believe direct contact with MgO nanoparticles

was important for bactericidal effect; however, further research is necessary to determine the exact mechanisms to take full advantages of MgO nanoparticles for antimicrobial applications.

5. Conclusions

This article reported the dose-dependent responses of BMSC, *E. coli*, and *S. epidermidis* toward MgO nanoparticles for the first time. The two *in vitro* culture methods used, i.e., co-seeding and sequential-seeding, affected the BMSC response at low concentrations of MgO nanoparticles. MgO nanoparticles at a concentration of 200 µg/mL enhanced BMSC adhesion density and proliferation up to 72 hours *in vitro* when sequentially-seeded. MgO nanoparticle concentrations at or above 500 µg/mL caused near to complete BMSC death independent of the culture methods. The bacterial culture showed that MBC of MgO nanoparticles for *E. coli* and *S. epidermidis* was 1200 µg/mL, and reduced bacterial adhesion was observed at and above 200 µg/mL of MgO nanoparticles. The exact mechanisms for the bactericidal effects of MgO nanoparticles remained elusive, but most likely due to direct-contact induced cell wall/membrane damage. Collectively, these *in vitro* results suggested that MgO nanoparticles at a concentration of 200 µg/mL provided dual benefits of promoting BMSC proliferation while reducing bacterial adhesion, which should be further studied for potential medical applications. The use of free MgO nanoparticles yielded detrimental effects to BMSCs in concentrations above 300µg/mL. We recommend further study into MgO nanoparticle as a coating material or as a part of a composite.

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Figure 1: Characterization of MgO nanoparticles. (A) SEM image, (B) TEM image, (C) EDS analysis, (D) particle size distribution, and (E) XRD spectrum.

Figure 2: Morphology and adhesion density of BMSCs that were co-seeded into DMEM with MgO nanoparticles at concentrations of 200 – 2000 μg/mL. (A-C) Fluorescence images of BMSCs after 24 hours of incubation with MgO nanoparticles at a concentration of (A) 200 μg/mL and (B) 300 μg/mL, respectively, as compared with (C) the Cells Only control that did not have any nanoparticles in culture. BMSCs were seeded at a density of 10,000 cells/cm² at time zero (t₀) as indicated by dashed line and incubated for 24 hours with 200 to 2000 μg/mL of MgO nanoparticles. Examples of cells with abnormal morphology are circled with a dashed yellow line. Blue stain indicates nuclei and green stain indicates F-actin. (D) BMSC adhesion density quantified from fluorescence images. Values are mean ± standard error; n=30. **p*<0.05 compared to the Cells Only control, ***p*<0.05 compared to 300-2000 μg/mL.

Figure 3: Media analyses after co-seeding and 24 hours of incubation of BMSCs with MgO nanoparticles at concentrations of 200 – 2000 $\mu\text{g/mL}$. (A) post-culture media pH, (B) Mg^{2+} ion concentration in the post-culture media, and (C) Ca^{2+} ion concentration in the post-culture media. Red dashed line indicates the linear fit for the data corresponding to 200-2000 $\mu\text{g/mL}$ MgO

nanoparticles. Values are mean $\pm$ standard deviation; n=3. * p <0.05 compared to the Cells Only control.

Figure 4: Fluorescence images of BMSCs that were sequentially cultured in DMEM with MgO nanoparticles at concentrations of (A-D) 200 – 700 µg/mL for (A-D) 24, (A'-D') 48, and (A''-D'') 72 hours compared to (E-E'') the respective Cells Only control at each time point. MgO

nanoparticles at concentrations of 1000 - 2000 $\mu\text{g/mL}$ caused complete cell death and images are not shown since no viable cells were found. The Cells Only group did not have any MgO nanoparticles. Examples of cells with abnormal morphology are circled with a dashed yellow line. Blue stains nuclei and green stains F-actin. Scale bar = 100 μm .

Figure 5: Adhesion density of BMSCs after they were sequentially cultured in DMEM with MgO nanoparticles at concentrations of 200 – 2000 µg/mL for 24, 48, and 72 hours, quantified from fluorescence images. BMSCs were seeded at a density of 10,000 cells/cm² at time zero (t₀) as indicated by dashed line. The cell density after incubation for 24 hours but prior to exposure to MgO is represented by the t₂₄ group. Afterwards, 200 to 2000µg/mL of MgO nanoparticles were added into the BMSC culture and incubated for additional 24, 48, 72 hours. Values are mean ± standard error; n=30. **p*<0.05 compared to the Cells Only control for respective time points, +*p*<0.05 compared to the same MgO concentration group at the earlier time points of 24h+24h and 24h+48h, as well as all the other groups with greater than 200 µg/mL MgO concentrations and the Cells Only control at all time points, ++*p*<0.05 compared to the same MgO concentration group at the earlier time point of 24h+24h, as well as all the groups with greater than 300 µg/mL MgO concentrations at all of the time points, #*p*<0.05 compared to the Cells Only control group at 24h+24h and 24h+48h time points.

Figure 6: Media analyses after BMSCs were sequentially cultured with MgO nanoparticles at concentrations of 200 – 2000 $\mu\text{g/mL}$ for 24, 48, and 72 hours. (A) post-culture media pH at each prescribed MgO concentration and each prescribed time point, (B) Mg^{2+} ion concentration in the post-culture media at each prescribed MgO concentration and each prescribed time point, and

(C) Ca^{2+} ion concentration in the post-culture media at each prescribed MgO concentration and each prescribed time point. Green dashed line indicates linear fit for the data corresponding to the 24h+24h time point with 200-2000 $\mu\text{g/mL}$ MgO. Values are mean $\pm$ standard deviation; $n=3$. * $p<0.05$ compared to the Cells Only control at respective time points, ** $p<0.05$ compared to 1400-2000 $\mu\text{g/mL}$ MgO, # $p<0.05$ compared to 24h+24h time point at respective MgO concentration, ## $p<0.05$ compared to 24h+48h time point at respective MgO concentration.

Figure 7: Media analyses after 24 hours of incubation of DMEM with MgO nanoparticles at concentrations of 200 – 2000 µg/mL at acellular condition. (A) post-culture media pH, (B) Mg²⁺ ion concentration in the post-culture media, and (C) Ca²⁺ ion concentration in the post-culture media. Black dashed line indicates the linear fit for the data corresponding to 200-2000 µg/mL MgO. Values are mean ± standard deviation, n=3. **p*<0.05 compared to the Media Only Control.

Figure 8: The effect of MgO nanoparticles on the pH of DMEM over 24 hours at acellular condition. Values are mean $\pm$ standard deviation; $n=3$ * $p<0.05$ compared to the Media Only control at respective time points, # $p<0.05$ compared to 0 minute time point of respective concentration group, ## $p<0.05$ compared to 24 hour time point of respective concentration group.

Figure 9: SEM images of (A⁻-E⁻) *E. coli* and (A⁺-E⁺) *S. epidermidis* after cultured with MgO nanoparticles at concentrations of (A-D) 200 – 700 $\mu\text{g/mL}$ and (E) Bacteria Only control without MgO nanoparticles. MgO nanoparticles at concentrations of 1000 - 2000 $\mu\text{g/mL}$ caused

complete bacterial death and thus images are not shown since no viable bacteria were found.
Scale bar = 2 μm .

Figure 10: Bacterial density after they were cultured in respective LBB and TSB with MgO nanoparticles at concentrations of 200 – 2000 µg/mL for 24 hours, as quantified from colony forming unit (CFU). Bacteria were seeded at a concentration of 5×10^5 bacteria/mL as indicated by the black dashed line. Values are mean $\pm$ standard deviation; n=9. * $p < 0.05$ compared to the Bacteria Only control of respective bacteria type, ** $p < 0.05$ compared to 1200-2000 µg/mL for *E. coli* or compared to 500-2000 µg/mL for *S. epidermidis*.

Figure 11: Broth analyses after bacteria were cultured in respective LBB and TSB with MgO nanoparticles at concentrations of 200 – 2000 µg/mL for 24 hours. (A) post-culture broth pH at each prescribed MgO concentration, (B) Mg²⁺ ion concentration in the post-culture broth at each prescribed MgO concentration, and (C) Ca²⁺ ion concentration in the post-culture broth at each

prescribed MgO concentration. A short-dashed line and long-dashed line indicate the linear fit for the data corresponding to 200-2000 $\mu\text{g/mL}$ MgO for *E. coli* and *S. epidermidis*, respectively. Values are mean $\pm$ standard deviation; n=3. * $p<0.05$ compared to the Bacteria Only control of respective bacteria type.

Figure 12: Broth analyses after MgO nanoparticles at concentrations of 200 – 2000 µg/mL were cultured in respective LBB and TSB for 24 hours without bacteria. (A) post-culture broth pH at each prescribed MgO concentration, (B) Mg²⁺ ion concentration in the post-culture broth at each prescribed MgO concentration, and (C) Ca²⁺ ion concentration in the post-culture broth at each prescribed MgO concentration. A short-dashed line and a long-dashed line indicate the linear fit for the data corresponding to 200-2000 µg/mL MgO for LBB and TSB, respectively. Values are

mean $\pm$ standard deviation; n=3. * p <0.05 compared to the Broth Only control for respective broth type, ** p <0.05 compared to 700-2000 $\mu\text{g/mL}$ MgO in respective broth type.

Figure 13: Bacterial density after they were cultured in respective LBB and TSB supplemented with 0 –50 mM Mg^{2+} ions for 24 hours, as quantified from colony forming unit (CFU). Bacteria were seeded at a concentration of 5×10^5 bacteria/mL as indicated by the black dashed line. Values are mean $\pm$ standard deviation; $n=9$. * $p<0.05$ compared to the control without supplemental Mg^{2+} dosage (0 mM), for respective bacteria type.

Figure 14: Broth analyses after bacteria were cultured in respective LBB and TSB supplemented with 0-50mM Mg^{2+} for 24 hours. (A) post-culture broth pH at each prescribed supplemental Mg^{2+} dosage, (B) Mg^{2+} ion concentration in the post-culture broth at each prescribed supplemental Mg^{2+} dosage, and (C) Ca^{2+} ion concentration in the post-culture broth at each prescribed supplemental Mg^{2+} dosage. A short-dashed line indicates the linear fit for the data corresponding to 1-50mM supplemental Mg^{2+} dosage in graph B and 1-40mM supplemental Mg^{2+} dosage in graph C for *E. coli*. A long-dashed line indicates the linear fit for the data

corresponding to 1-50mM supplemental Mg^{2+} dosage for *S. epidermidis*. Values are mean $\pm$ standard deviation; n=3. * $p < 0.05$ compared to the control without supplemental Mg^{2+} dosage (0 mM), for respective bacteria type.

Figure 15: Bacterial density after they were cultured for 24 hours in respective LBB and TSB with initial pH adjusted to 7-10, as quantified from colony forming unit (CFU). Bacteria were seeded at a concentration of 5×10^5 bacteria/mL as indicated by the black dashed line. Values are mean $\pm$ standard deviation; n=9. * $p < 0.05$ compared to the control with an initial pH 7 for respective bacteria type.

Figure 16: Broth analyses after bacteria were cultured for 24 hours in respective LBB and TSB with initial pH adjusted to 7-10. (A) post-culture broth pH at each prescribed initial pH, (B) Mg²⁺ ion concentration in the post-culture broth at each prescribed initial pH, and (C) Ca²⁺ ion concentration in the post-culture broth at each prescribed initial pH. Values are mean $\pm$ standard deviation; n=3. * $p < 0.05$ compared to the control with an initial pH 7 for respective bacteria type.

Figure 17: Summary diagram of critical MgO concentrations and their effects on BMSCs, *E. coli*, and *S. epidermidis*.

A

B

C

Figure 18: Correlation plots relating the post culture Mg^{2+} concentration to (A) BMSC density, (B) *E. coli* CFU and (C) *S. epidermidis* CFU after respective culture with MgO nanoparticles. (A) Data from Figure 5 and 6B. (B, C) Data from Figure 10 and 11B, Values are mean $\pm$ standard deviation.