

# Does Vitamin D3 Prevent the Inhibitory Effect of Vancomycin on Osteoblasts?

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## Abstract

**Background** The utility of vancomycin powder to prevent surgical site infection, mainly in spinal surgery, has been widely examined, and the local administration of vancomycin powder to wounds has been reported to be effective in preventing surgical site infections after spine surgery. However, in vitro studies have shown that high local concentrations of vancomycin may inhibit osteogenesis, although it remains unclear how these high concentrations

influence osteoblasts. No candidate drug has been reported to recover cytotoxicity with high concentrations of vancomycin, but we suggest that vitamin D3, which induces osteoblast proliferation, may be administered concomitantly with vancomycin in these situations.

**Questions/purposes** (1) Does a high concentration of vancomycin reduce viable osteoblast numbers in cell culture compared with controls? (2) Does vitamin D3 administration confer a protective effect on osteoblasts when administered with continuous vancomycin? (3) Does vitamin D3 administration confer a protective effect on osteoblasts when administered with pulsed vancomycin (24 hours of administration)? (4) Does vitamin D3 administration confer alkaline phosphatase, mineralization, and gene expression when administered with pulsed vancomycin?

**Methods** MC3T3-E1 cells were cultured at 37° C in an  $\alpha$ -minimum essential medium supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO<sub>2</sub>. The experimental concentrations of vancomycin (2500  $\mu$ g/mL, 5000  $\mu$ g/mL, and 7500  $\mu$ g/mL) were determined based on previous reports and preliminary experiments. We concomitantly administered vitamin D3 (0.01 nM) to prevent cytotoxicity in osteoblasts, using two different treatments: continuous vancomycin administration (measured at 6 hours, 12 hours, 24 hours, and 72 hours) and pulsed vancomycin for 24 hours (measured at 1 days, 3 days, and 7 days). We analyzed cell numbers and morphologic changes in cells treated with vancomycin or vancomycin plus 0.01 nM vitamin D3. Osteoblast differentiation was assessed with alkaline phosphatase staining, alkaline phosphatase activity, and Alizarin red S staining. **Results** The number of cells was reduced at 6 hours, 24 hours, 48 hours, and 72 hours in response to continuous vancomycin administration at 7500  $\mu$ g/mL (at 72 hours, control  $14.6 \times 10^4$  cells/mL  $\pm$   $0.260 \times 10^4$  cells/mL,

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One or more of the authors certifies that they (KT, SK, KT, HT) have a patent pending for a product that will prevent the inhibitory effect of vancomycin on osteogenesis.

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vancomycin at  $0.917 \times 10^4$  cells/mL  $\pm$   $0.288 \times 10^4$  cells/mL, mean difference  $-13.7 \times 10^4$  cells/mL  $\pm$   $0.388 \times 10^4$  cells/mL [95% CI -14.5 to -12.9];  $p < 0.001$ ). Vitamin D3 did not have a protective effect when vancomycin was administered continuously at 7500  $\mu$ g/mL (at 72 hours, vancomycin alone  $0.917 \times 10^4$  cells/mL  $\pm$   $0.288 \times 10^4$  cells/mL, vancomycin + vitamin D3  $1.67 \times 10^4$  cells/mL  $\pm$   $0.310 \times 10^4$  cells/mL, mean difference  $0.75 \times 10^4$  cells/mL  $\pm$   $0.423 \times 10^4$  cells/mL [95% CI -0.127 to 1.63];  $p = 0.09$ ). With pulsed administration for only the first 24 hours, the number of cells was reduced at 1 day, 3 days, and 7 days at 7500  $\mu$ g/mL (at 7 days, control  $18.6 \times 10^4$  cells/mL  $\pm$   $1.29 \times 10^4$  cells/mL, vancomycin at  $3.46 \times 10^4$  cells/mL  $\pm$   $0.292 \times 10^4$  cells/mL, mean difference  $-15.1 \times 10^4$  cells/mL  $\pm$   $1.33 \times 10^4$  cells/mL [95% CI -17.9 to -12.4];  $p < 0.001$  for all). However, vitamin D3 had a recovery effect when vancomycin was administered only for 24 hours (cell number with 7500  $\mu$ g/mL, day 7: vancomycin alone  $3.46 \times 10^4$  cells/mL  $\pm$   $0.292 \times 10^4$  cells/mL, vancomycin + vitamin D3  $10.6 \times 10^4$  cells/mL  $\pm$   $0.900 \times 10^4$  cells/mL, mean difference  $7.13 \times 10^4$  cells/mL  $\pm$   $0.946 \times 10^4$  cells/mL [95% CI 5.16 to 9.09];  $p < 0.001$ ). With the addition of vitamin D3, we observed recovery of alkaline phosphatase staining and Alizarin red staining (evidence of calcification) but no difference in the gene expression of Type I collagen (vancomycin alone  $0.319 \pm 0.0730$ , vancomycin + vitamin D3  $0.511 \pm 0.139$ , mean difference  $0.192 \pm 0.157$  [95% CI -0.483 to 0.867];  $p = 0.345$ ), alkaline phosphatase (vancomycin alone  $0.532 \pm 0.0210$ , vancomycin + vitamin D3  $0.785 \pm 0.0590$ , mean difference  $0.253 \pm 0.0620$  [95% CI -0.0150 to 0.521];  $p = 0.0550$ ), and cathelicidin antimicrobial peptide (vancomycin alone  $0.885 \pm 0.0520$ , vancomycin + vitamin D3  $1.24 \pm 0.125$ , mean difference  $0.355 \pm 0.135$  [95% CI -0.0200 to 0.730];  $p = 0.0580$ ).

**Conclusion** We found that 7500  $\mu$ g/mL of vancomycin is cytotoxic to osteoblasts. Cytotoxicity could be prevented by administering vitamin D3 in combination with vancomycin.

**Clinical Relevance** The high concentrations of vancomycin routinely used clinically raises concerns related to osteoblast cytotoxicity, which may contribute to pseudoarthrosis after spinal surgery. Thus, vitamin D3, which is frequently used to treat osteoporosis, may have efficacy as a concomitantly administered drug by inducing the proliferation of osteoblasts. These results indicate that a combination therapy of vancomycin and vitamin D3 may prevent adverse events such as osteoblast cytotoxicity.

## Introduction

Antimicrobial prophylaxis is useful for preventing infection after spinal surgery. Conventionally, cefazolin,

an antibiotic that targets *Staphylococcus aureus*, is used for antimicrobial prophylaxis. However, the incidence of surgical site infections caused by methicillin-resistant *S. aureus* and coagulase-negative *Staphylococcus* has recently increased. In these cases, vancomycin has been used for antimicrobial prophylaxis to combat methicillin-resistant *S. aureus* and coagulase-negative *Staphylococcus* infections [10, 11, 13]. The systemic delivery of vancomycin is, however, subject to certain difficulties [19, 22]. For example, when administering vancomycin systemically, it is difficult to deliver optimal concentrations at the surgical site. In addition, vancomycin administration is sometimes associated with adverse events, including hypotension, flushing, rashes, colitis, and Stevens-Johnson syndrome. To circumvent these issues, the utility of a topical vancomycin powder to prevent surgical site infections, mainly in spinal surgery, has been widely examined. The local administration of vancomycin powder to wounds is an effective method of preventing surgical site infections after spinal surgery [9, 23, 24]. Adverse events associated with the administration of high concentrations of vancomycin may occur very rarely in spinal surgery. To date, however, pseudoarthrosis because of the local application of vancomycin powder has yet to be reported. This may be attributable to the fact that there has been only short-term follow-up and limited radiographic imaging to confirm successful fusion [23]. Furthermore, two studies have reported that high concentrations of vancomycin may have a cytotoxic effect on osteoblast cells [5, 6]. Although the administration of high-dose vancomycin at surgical sites has been reported to cause cytotoxicity [5, 6], lower concentrations of vancomycin may not prevent surgical site infection. Thus, the use of concomitantly administered drugs that promote osteogenesis could be one strategy to ameliorate high-dose vancomycin-induced cytotoxicity in osteoblasts. Vitamin D3, which is used to treat osteoporosis, is effective in clinical practice, not only in suppressing fractures but also in inducing osteoblast proliferation. Therefore, vitamin D3 is a candidate drug that could be administered concomitantly with vancomycin.

Therefore, we asked: (1) Does a high concentration of vancomycin reduce viable osteoblast numbers in cell culture compared with controls? (2) Does vitamin D3 administration confer a protective effect on osteoblasts when administered with continuous vancomycin? (3) Does vitamin D3 administration confer a protective effect on osteoblasts when administered with pulsed vancomycin (24 hours of administration)? (4) Does vitamin D3 administration confer alkaline phosphatase, mineralization, and gene expression when administered with pulsed vancomycin?

## Materials and Methods

### Cell Culture

MC3T3-E1 cells derived from mouse calvaria were provided by RIKEN BRC, which is participating in the National Bio-Resource Project of the MEXT/AMED, Japan. MC3T3-E1 cells have a rate of proliferation similar to other osteoblast cell lines, and their matrices are mineralized in a similar manner [3, 4]. MC3T3-E1 cells were cultured in an  $\alpha$ -minimum essential medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Co, Ltd, Tokyo, Japan) and 1% antibiotics (100 U/mL of penicillin and 100  $\mu$ g/mL of streptomycin) (Wako Pure Chemical Corp, Osaka, Japan) in a humidified incubator containing 5% CO<sub>2</sub> at 37° C. Cells were passaged when reaching 70% confluency. After the cells were washed with phosphate-buffered saline, they were treated with trypsin-EDTA (0.05% trypsin, EDTA-4Na; Thermo Fisher Scientific) and incubated at 37° C for 5 minutes, after which they were detached and cultured in fresh flasks.

### Vancomycin Concentrations for In Vitro Experiments

The vancomycin used in this study was purchased from Shionogi & Co, Ltd (Osaka, Japan). The vancomycin material was dissolved in a 5%  $\alpha$ -minimum essential medium, and an unmodified medium was used as a vehicle control. We examined the effects of vancomycin at concentrations ranging from 2500  $\mu$ g/mL to 7500  $\mu$ g/mL, based on the concentrations used in clinical practice (surgical drainage). When 2 g of vancomycin are applied topically, it has been estimated that the average scoliosis would hold approximately 1500  $\mu$ g/mL of fluid [24]. Based on a past study, we assumed that concentrations of vancomycin used clinically, ranging from 0.5 g to 6 g, would correspond to 2500  $\mu$ g/mL to 7500  $\mu$ g/mL of vancomycin in vitro [24].

### Vitamin D3 Concentration

The vitamin D3 used in the present study was purchased from Cayman Chemical (Ann Arbor, MI, USA). The experimental concentrations of vitamin D3 were determined based on previous reports and preliminary experiments [12, 15, 27]. Kim et al. [13] reported that vitamin D3 concentrations ranging from 0.001 nM to 0.1 nM influence cell differentiation and matrix mineralization. In preliminary experiments, we examined cell numbers and morphologic changes in response to vitamin

D3 treatment at concentrations of 0.01 nM, 1 nM, and 100 nM, and observed cytotoxicity at 100 nM (data not shown). For the present study, we therefore selected a 0.01 nM concentration of vitamin D3. Before experimentation, vitamin D3 was dissolved in a 5%  $\alpha$ -minimum essential medium, and an unmodified medium was used as a vehicle control.

### Cell Number and Morphology in Groups Treated with Continuous Vancomycin and Continuous Vancomycin Plus 0.01 nM Vitamin D3

MC3T3-E1 cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL and grown for approximately 3 days until reaching 70% confluency. The cells were subsequently divided into four treatment groups: 2500  $\mu$ g/mL, 5000  $\mu$ g/mL, and 7500  $\mu$ g/mL of vancomycin and a control group. The effect of continuous administration of high-dose vancomycin on the number of osteoblast cells and their morphologic changes were examined at 6 hours, 24 hours, 48 hours, and 72 hours post-administration. The effect of continuous administration of high-dose vancomycin and vitamin D3 on the number of osteoblast cells and their morphologic changes were investigated at 6 hours, 24 hours, 48 hours, and 72 hours post-administration. Thereafter, the cells were divided into four groups: 2500  $\mu$ g/mL vancomycin + 0.01 nM vitamin D3, 5000  $\mu$ g/mL vancomycin + 0.01 nM vitamin D3, 7500  $\mu$ g/mL vancomycin + 0.01 nM vitamin D3, and 0.01 nM vitamin D3. For cell counting, cells were detached using 0.25% trypsin-EDTA. Viable cell numbers were determined using a Trypan blue dye exclusion test. To analyze cell proliferation and morphologic changes, we used an Olympus IX70 microscope (Olympus Corp, Tokyo, Japan).

### Cell Number and Morphology in Groups Treated with Pulsed Vancomycin and Pulsed VCM Plus 0.01 nM Vitamin D3

MC3T3-E1 cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL and grown for approximately 3 days until reaching 70% confluency. The cells were subsequently divided into four treatment groups: 2500  $\mu$ g/mL, 5000  $\mu$ g/mL, and 7500  $\mu$ g/mL of vancomycin and a control group. To determine the influence of high-dose vancomycin pulse exposure on cytotoxicity in osteoblast cells, we cultured the cells in the presence of vancomycin for 24 hours. In clinical practice, the concentration of vancomycin at the surgical site has been observed to reach a maximum value within 24 hours, after which the concentration decreases [17].

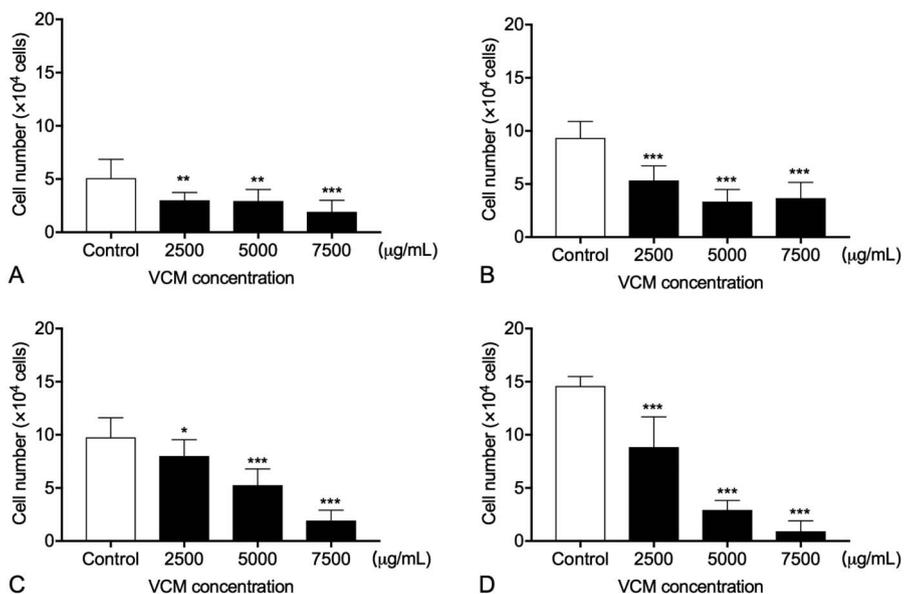
Thereafter, vancomycin was removed and cells were cultured in the  $\alpha$ -minimum essential medium supplemented with 5% fetal bovine serum for 1 day, 3 days, or 7 days. To examine the cytotoxic effects of pulse exposure to high-dose vancomycin and vitamin D3, we cultured osteoblast cells in the presence of vancomycin and vitamin D3 for 24 hours, after which the cells were cultured with 0.01 nM of vitamin D3 for 1 day, 3 days, and 7 days. The cell number was compared between the vancomycin and vancomycin plus vitamin D3 groups. The proliferation of and morphologic changes in these cells were examined using an Olympus IX70 microscope (Olympus Corp).

### Assessment of Alkaline Phosphatase Staining and Activity

#### Alkaline Phosphatase Staining

Treated MC3T3-E1 cells were stained for the presence of alkaline phosphatase using an alkaline phosphatase staining kit (Cosmo Bio Ltd, Tokyo, Japan). The MC3T3-E1 cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL and grown for approximately 3 days until reaching 70% confluency. The experiments were conducted using vancomycin concentrations that had the most combined effects. To

evaluate the protective effect of vitamin D3 on osteoblast cells treated with vancomycin, we measured alkaline phosphatase activity at day 7 using the vancomycin concentration that had the most pronounced detrimental effect (7500  $\mu\text{g/mL}$ ). When the cells reached pre-confluence, they were divided into the following three groups: 7500  $\mu\text{g/mL}$  vancomycin, 7500  $\mu\text{g/mL}$  vancomycin + 0.01 nM vitamin D3, and a control. Cells in the vancomycin and vancomycin + vitamin D3 groups were exposed for 24 hours, after which vancomycin was removed. The cells in the vancomycin group were then cultured in an  $\alpha$ -minimum essential medium supplemented with 5% fetal bovine serum, whereas cells in the vancomycin + vitamin D3 group were cultured in a medium containing 0.01 nM of vitamin D3. After 7 days, alkaline phosphatase staining was performed. After removing the culture solution, we washed the wells three times with 1 mL of phosphate-buffered saline, followed by the addition of a 200  $\mu\text{L}$ /well of fixative solution (10% neutral buffered formalin) and cells were fixed for 20 minutes at room temperature. Thereafter, the fixative was removed, and the cells were washed three times with 2 mL of distilled water per well. Five milliliters of substrate buffer was added to the chromogenic substrate, and 200  $\mu\text{L}$  of the resulting mixture was added per well, followed by incubation at 37°C for 20 minutes. Alkaline phosphatase activity was indicated by color development of the blue dye. After a sufficient signal was obtained, the



**Fig. 1A-D** This figure shows the dose- and time-dependent effects of vancomycin on the growth of MC3T3-E1 osteoblast cells. Cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL. The cells were cultured with various concentrations of vancomycin for (A) 6 hours, (B) 24 hours, (C) 48 hours, and (D) 72 hours. Error bars show the mean  $\pm$  SD. The data represent three independent experiments; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; VCM = vancomycin.

walls of the wells were washed with distilled water to terminate the reaction.

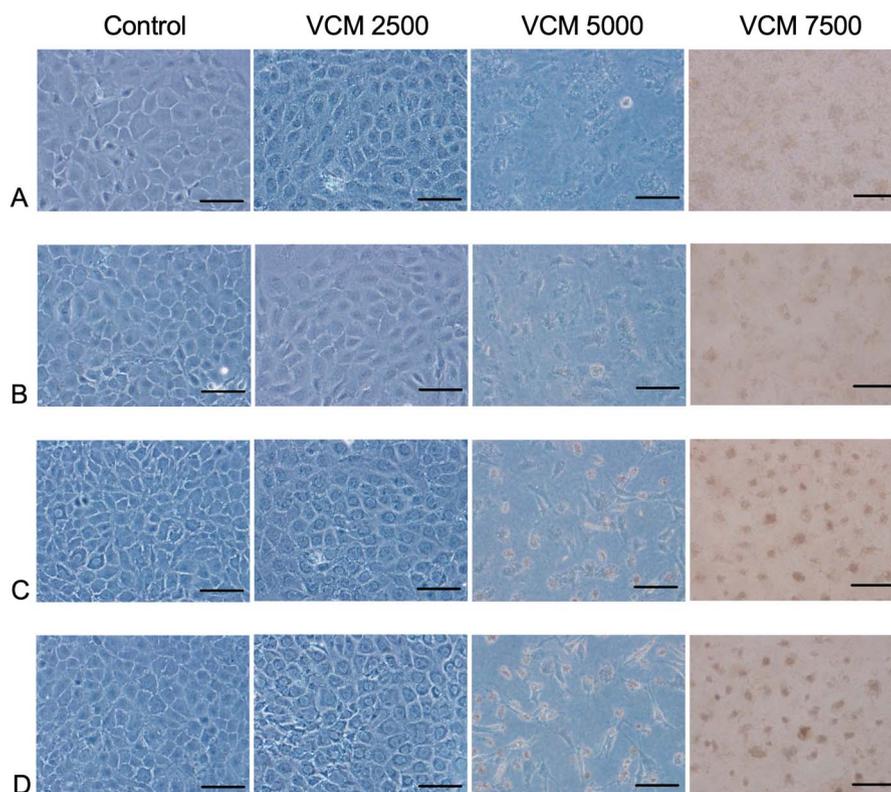
#### Alkaline Phosphatase Activity Assay

An assay for alkaline phosphatase activity was performed using an alkaline phosphatase kit (Wako Pure Chemical Industries) according to the manufacturer's instructions. Briefly, MC3T3-E1 cells were added to the wells of a 96-well plate at a density of  $1.0 \times 10^4$  cells/mL and divided into the same treatment groups used for alkaline phosphatase staining. Alkaline phosphatase activity was measured after incubation for 7 days. For the assay, 100  $\mu$ L of substrate was added to each well, and 20  $\mu$ L of cell supernatant that was obtained from the control, vancomycin, and vancomycin + vitamin D3 groups was added to each well. After agitating the cells for 1 minute using a plate mixer, we incubated the cells at 37° C for 15 minutes. The reaction was then terminated by adding 80  $\mu$ L of stop solution to each well, and after agitation for 1 minute using a

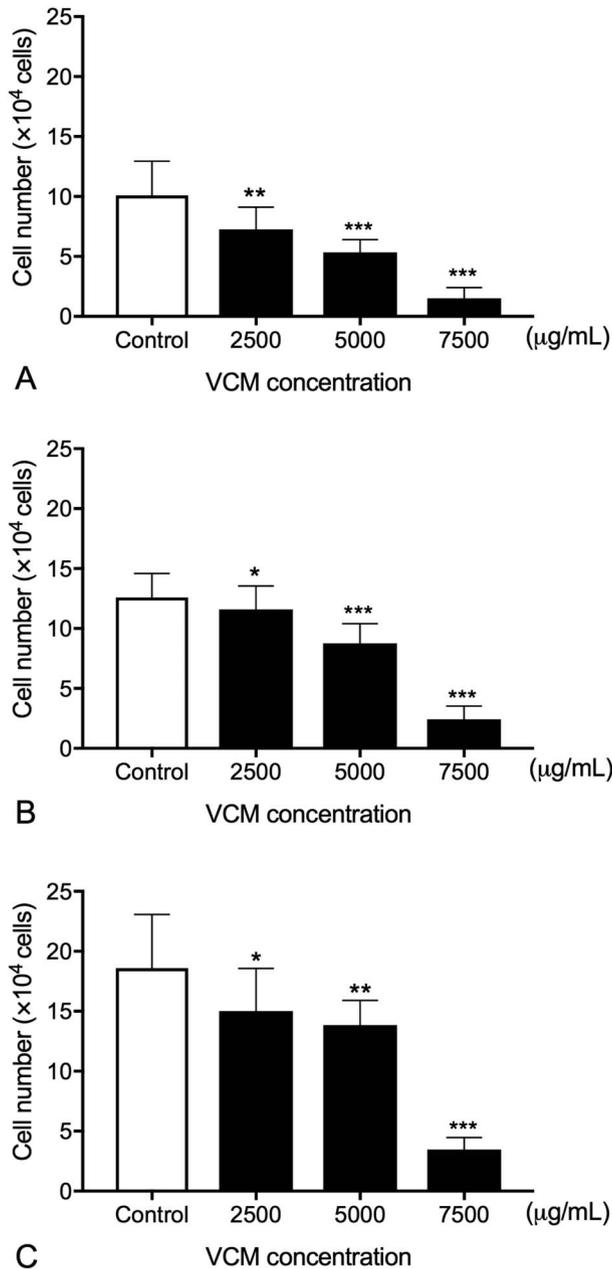
plate mixer, absorbance was measured at 405 nm using a microplate reader.

#### Alizarin Red Staining

To evaluate the mineralization of MC3T3-E1 cells, we performed Alizarin red S staining using paraformaldehyde and Alizarin red S solutions purchased from Sigma-Aldrich (St. Louis, MO, USA). Specifically, we analyzed differences in calcified nodules produced in response to treatment with 7500  $\mu$ g/mL vancomycin and 7500  $\mu$ g/mL vancomycin + 0.01 nM vitamin D3. MC3T3-E1 cells were added to the wells of a 12-well plate at a density of  $1.0 \times 10^4$  cells/mL. For cells cultured for a longer period, the medium was changed at 2- to 3-day intervals. After 28 days, the medium was removed, and the cells were given three 5-minute washes with 1 mL of phosphate-buffered saline. Thereafter, the cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, and the fixation solution



**Fig. 2A-D** This figure shows dose- and time-dependent morphologic changes in MC3T3-E1 osteoblast cells after the administration of vancomycin. Cells were added to a flask at a density of  $1.0 \times 10^4$  cells/mL. The cells were cultured with various concentrations of vancomycin for (A) 6 hours, (B) 24 hours, (C) 48 hours, and (D) 72 hours. Cell proliferation and morphologic changes were examined using a light microscope. The data represent three independent experiments. Scale bars represent 50  $\mu$ m; VCM = vancomycin.



**Fig. 3A-C** After the administration of vancomycin for 24 hours, its effects on the growth of MC3T3-E1 osteoblast cells were measured. Cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL. The cells were exposed to various concentrations of vancomycin for 24 hours. Thereafter, vancomycin was removed and the cells were cultured in an  $\alpha$ -minimum essential medium supplemented with 5% fetal bovine serum for (A) 1 day, (B) 3 days, and (C) 7 days. Error bars show the mean  $\pm$  SD. The data represent three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; VCM = vancomycin.

was removed by washing it with distilled water (two 10-minute washes). The cells were stained with 2% Alizarin red S (pH 4.2) for 10 minutes at room temperature, with

minimal light exposure. The stained cells were subsequently washed once with phosphate-buffered saline, and macroscopic observations were performed using a light microscope.

### RNA Isolation and Gene Expression Analysis

Total RNA was isolated from osteoblast cells using the TRIzol Reagent (Thermo Fisher Scientific), according to the manufacturer's instruction. For the quantitative reverse transcription-polymerase chain reaction analysis, total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Data analysis using the SYBR Green real-time reverse transcription-polymerase chain reaction technique was performed with the 7500 Fast Real-Time Polymerase Chain Reaction System (Applied Biosystems). We used the following polymerase chain reaction primers: Type I collagen, 5'-CTGGCTTTGCCGCCC-3' (forward) and 5'-ACCTTTAACACCAGTATCACCAGGT-3' (reverse); alkaline phosphatase, 5'-GGAATACGAAGTGGATGAAGGCC-3' (forward) and 5'-CAGTTCAGTGCGGT-TCCAGACATAG-3' (reverse); camp, 5'-GCTGTGGCGGT-CACTATCAC-3' (forward) and 5'-TGTCTAGGGACTG-CTGGTTGA-3' (reverse); and 18S rRNA, 5'-GCAATTA-TTCCCCATGAACG-3' (forward) and 5'-GGCCTCACT-AAACCATCCAA-3' (reverse).

Relative fold changes in transcript levels were calculated using the  $2^{-\Delta\Delta CT}$  method (where  $CT$  is the threshold cycle), using the housekeeping gene that encodes 18S rRNA as a reference standard for the amount loaded and the quality of cDNA.

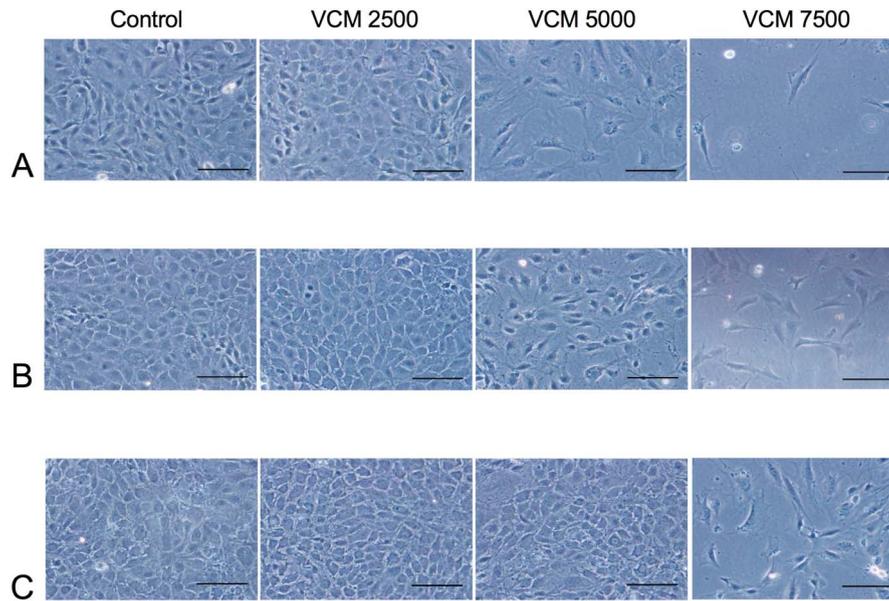
### Statistical Analysis

The statistical analysis was performed using Graph Pad Prism version 7.0 (San Diego, CA, USA). All error bars represent the SD. A t-test was used for comparisons between two groups, and ANOVA, followed by Tukey's multiple-comparison test, was performed for comparisons between more than two groups. A threshold of  $p < 0.05$  was set and taken to indicate statistical significance.

### Results

#### Influence of Continuous Exposure to High-dose Vancomycin on Cytotoxicity in Osteoblast Cells

The dose- and time-dependent effects of continuous administration of high-dose vancomycin on the number of



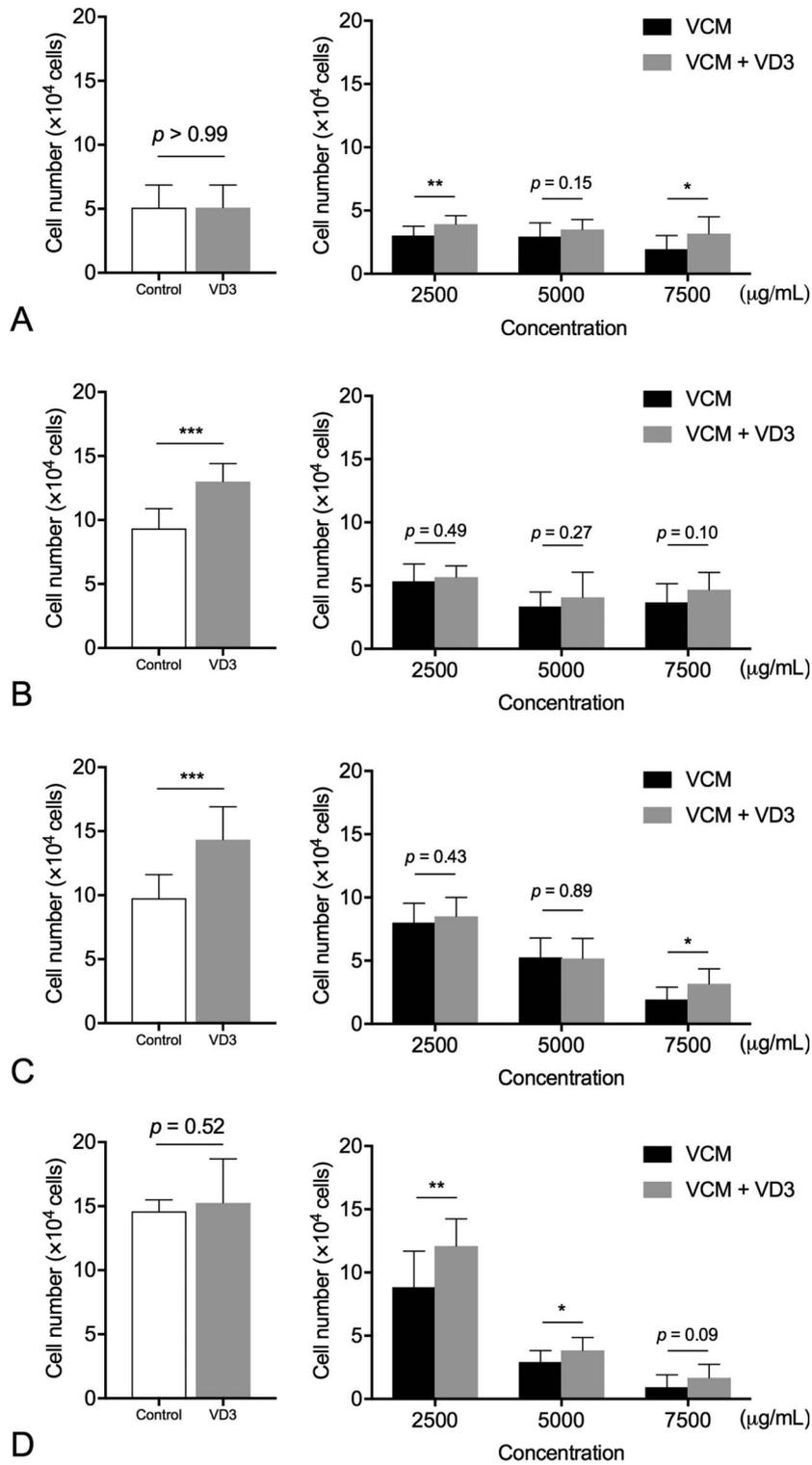
**Fig. 4A-C** This figure shows morphologic changes in MC3T3-E1 osteoblast cells in response to the administration of vancomycin for 24 hours. Cells were added to a flask at a density of  $1.0 \times 10^4$  cells/mL. The cells were exposed to various concentrations of vancomycin for 24 hours. Thereafter, vancomycin was removed and the cells were cultured in an  $\alpha$ -minimum essential medium supplemented with 5% fetal bovine serum for (A) 1 day, (B) 3 days, and (C) 7 days. Cell proliferation and morphologic changes were examined using a light microscope. The data represent three independent experiments. Scale bars represent 50  $\mu$ m; VCM = vancomycin.

osteoblast cells and their morphologic changes occurred at all timepoints and all concentrations. We found there was a dose-dependent decrease in the number of osteoblasts at 6 hours, 24 hours, 48 hours, and 72 hours after the administration of vancomycin (Fig. 1). The number of cells was reduced at 2500  $\mu$ g/mL (at 72 hours, control  $14.6 \times 10^4$  cells/mL  $\pm$   $0.260 \times 10^4$  cells/mL, vancomycin at  $0.883 \times 10^4$  cells/mL  $\pm$   $0.824 \times 10^4$  cells/mL, mean difference  $-5.75 \times 10^4$  cells/mL  $\pm$   $0.864 \times 10^4$  cells/mL [95% CI -7.54 to -3.96];  $p < 0.001$ ). The number of cells was also reduced at 5000  $\mu$ g/mL (at 72 hours, control  $14.6 \times 10^4$  cells/mL  $\pm$   $0.260 \times 10^4$  cells/mL, vancomycin at  $2.92 \times 10^4$  cells/mL  $\pm$   $0.260 \times 10^4$  cells/mL, mean difference  $-11.7 \times 10^4$  cells/mL  $\pm$   $0.368 \times 10^4$  cells/mL [95% CI -12.4 to -10.9];  $p < 0.001$ ). The number of cells was also reduced at 7500  $\mu$ g/mL (at 72 hours, control  $14.6 \times 10^4$  cells/mL  $\pm$   $0.260 \times 10^4$  cells/mL, vancomycin at  $0.917 \times 10^4$  cells/mL  $\pm$   $0.288 \times 10^4$  cells/mL, mean difference  $-13.7 \times 10^4$  cells/mL  $\pm$   $0.388 \times 10^4$  cells/mL [95% CI -14.5 to -12.9];  $p < 0.001$ ). In the control and 2500  $\mu$ g/mL vancomycin treatment groups, osteoblasts tended to show time-dependent development of spherical morphology (Fig. 2A-D), which indicated the absence of cytotoxicity. In contrast, no changes were observed in the morphology of cells treated with 5000  $\mu$ g/mL and 7500  $\mu$ g/mL of

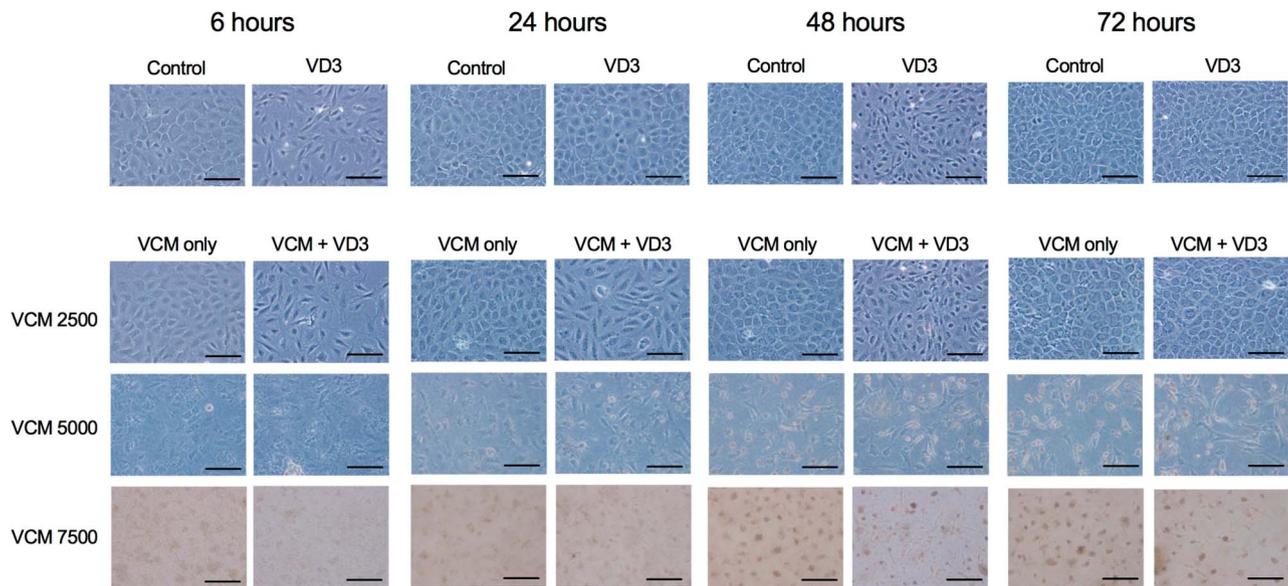
vancomycin from 6 hours to 72 hours after administration (Fig. 2A-D).

#### Influence of pulsed Exposure to High-dose Vancomycin on Cytotoxicity in Osteoblast Cells

With pulsed administration for only the first 24 hours, the number of cells was reduced at all time points and with all concentrations (Fig. 3A-C). The number of cells was reduced at 2500  $\mu$ g/mL (at 7 days, control  $18.6 \times 10^4$  cells/mL  $\pm$   $1.29 \times 10^4$  cells/mL, vancomycin at  $15.0 \times 10^4$  cells/mL  $\pm$   $1.03 \times 10^4$  cells/mL, mean difference  $-3.58 \times 10^4$  cells/mL  $\pm$   $1.65 \times 10^4$  cells/mL [95% CI -7.01 to -0.154];  $p < 0.01$ ). The number of cells was also reduced at 5000  $\mu$ g/mL (at 7 days, control  $18.6 \times 10^4$  cells/mL  $\pm$   $1.29 \times 10^4$  cells/mL, vancomycin at  $13.8 \times 10^4$  cells/mL  $\pm$   $0.601 \times 10^4$  cells/mL, mean difference  $-4.75 \times 10^4$  cells/mL  $\pm$   $1.43 \times 10^4$  cells/mL [95% CI -7.71 to -1.79];  $p < 0.01$ ). The number of cells was also reduced at 7500  $\mu$ g/mL (at 7 days, control  $18.6 \times 10^4$  cells/mL  $\pm$   $1.29 \times 10^4$  cells/mL, vancomycin at  $3.46 \times 10^4$  cells/mL  $\pm$   $0.292 \times 10^4$  cells/mL, mean difference  $-15.1 \times 10^4$  cells/mL  $\pm$   $1.33 \times 10^4$  cells/mL [95% CI -17.9 to -12.4];  $p < 0.001$ ). In the control, 2500  $\mu$ g/mL, and 5000  $\mu$ g/mL vancomycin treatment groups, osteoblasts tended to show



**Fig. 5A-D** This figure shows the effect of vitamin D3 in the presence of vancomycin on the number of MC3T3-E1 osteoblast cells. Cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL. The cells were cultured with various concentrations of vancomycin and 0.01 nM of vitamin D3 for **(A)** 6 hours, **(B)** 24 hours, **(C)** 48 hours, and **(D)** 72 hours. Error bars show the mean  $\pm$  SD. The data represent three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; VCM = vancomycin; VD3 = vitamin D3.



**Fig. 6** This figure shows the protective effects of vitamin D3 on the morphology of MC3T3-E1 osteoblast cells in the presence of vancomycin. Cells were added to a flask at a density of  $1.0 \times 10^4$  cells/mL. The cells were cultured with various concentrations of vancomycin with or without 0.01 nM of vitamin D3 for 6 hours, 24 hours, 48 hours, and 72 hours. Cell proliferation and morphologic changes were examined using a light microscope. The data represent three independent experiments. Scale bars represent 50  $\mu$ m; VCM = vancomycin; VD3 = vitamin D3.

time-dependent development of spherical morphology (Fig. 4A-C). These morphological changes indicate the absence of cytotoxicity. In contrast, no changes were observed in the morphology of cells treated with 7500  $\mu$ g/mL of vancomycin from 1 day to 7 days after administration (Fig. 4A-C).

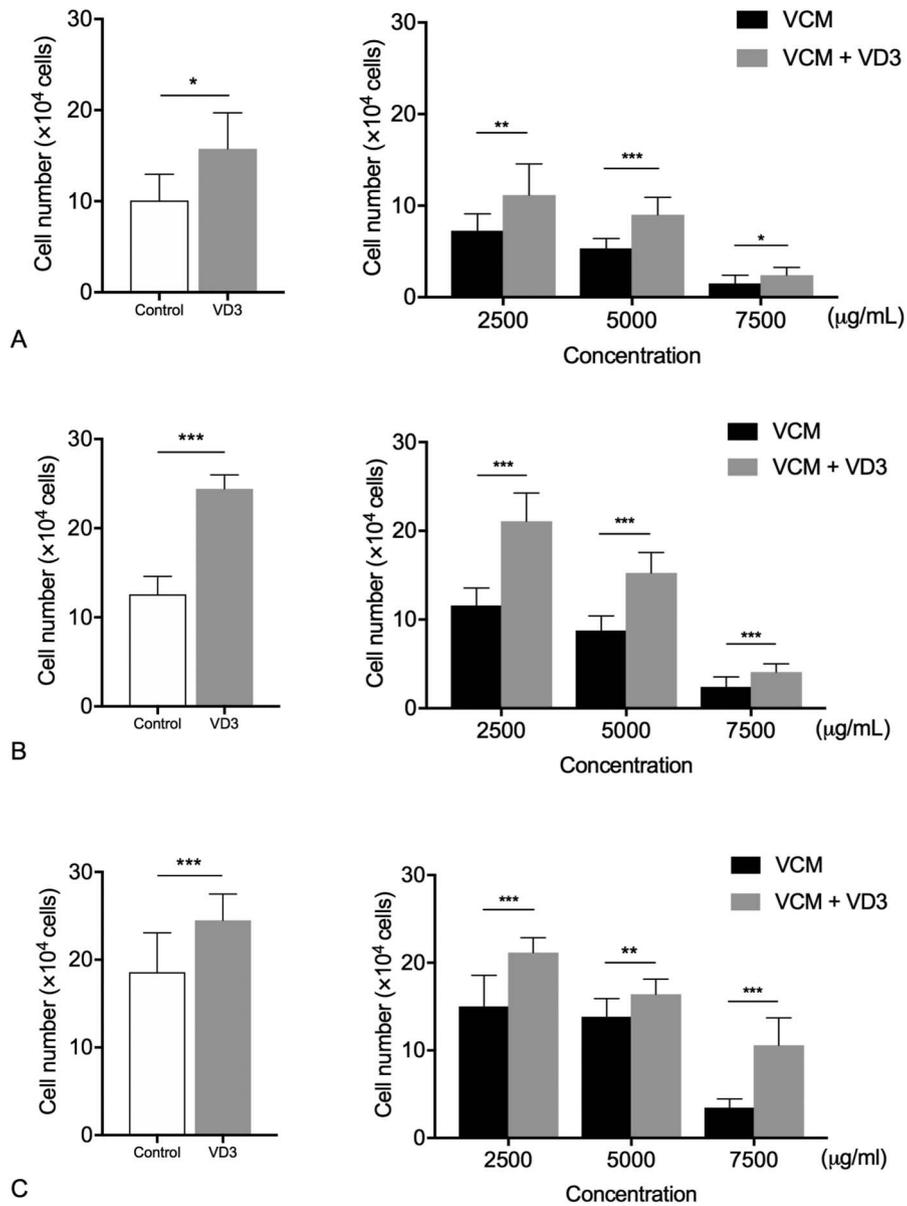
#### Effect of Vitamin D3 in Preventing Cytotoxicity in Osteoblast Cells Caused by Continuous Exposure to Vancomycin

The viability of osteoblast cells in the presence of vitamin D3 was no different from vancomycin administration alone for 72 hours (Fig. 5A-D). Vitamin D3 did not have a protective effect when vancomycin was administered continuously at 2500  $\mu$ g/mL (at 72 hours, vancomycin alone  $8.83 \times 10^4$  cells/mL  $\pm 0.824 \times 10^4$  cells/mL, vancomycin + vitamin D3  $12.1 \times 10^4$  cells/mL  $\pm 0.621 \times 10^4$  cells/mL, mean difference  $3.25 \times 10^4$  cells/mL  $\pm 1.03 \times 10^4$  cells/mL [95% CI 1.11 to 5.39];  $p < 0.01$ ). Vitamin D3 did not have a protective effect when vancomycin was administered continuously at 5000  $\mu$ g/mL (at 72 hours, vancomycin alone  $2.92 \times 10^4$  cells/mL  $\pm 0.260 \times 10^4$  cells/mL, vancomycin + vitamin D3  $3.83 \times 10^4$  cells/mL  $\pm 0.297 \times 10^4$  cells/mL, mean difference  $0.917 \times 10^4$  cells/mL  $\pm 0.394 \times 10^4$  cells/mL [95% CI 0.098 to 1.77];  $p < 0.05$ ). Vitamin D3 did not have a protective effect when vancomycin was

administered continuously at 7500  $\mu$ g/mL (at 72 hours, vancomycin alone  $0.917 \times 10^4$  cells/mL  $\pm 0.288 \times 10^4$  cells/mL, vancomycin + vitamin D3  $1.67 \times 10^4$  cells/mL  $\pm 0.310 \times 10^4$  cells/mL, mean difference  $0.750 \times 10^4$  cells/mL  $\pm 0.423 \times 10^4$  cells/mL [95% CI -0.127 to 1.63];  $p = 0.09$ ). In the microscopic examination, there was no observable difference between cells treated with vitamin D3 and those without (Fig. 6).

#### Effect of Vitamin D3 in Preventing Cytotoxicity in Osteoblast Cells Caused by Pulsed Exposure to Vancomycin

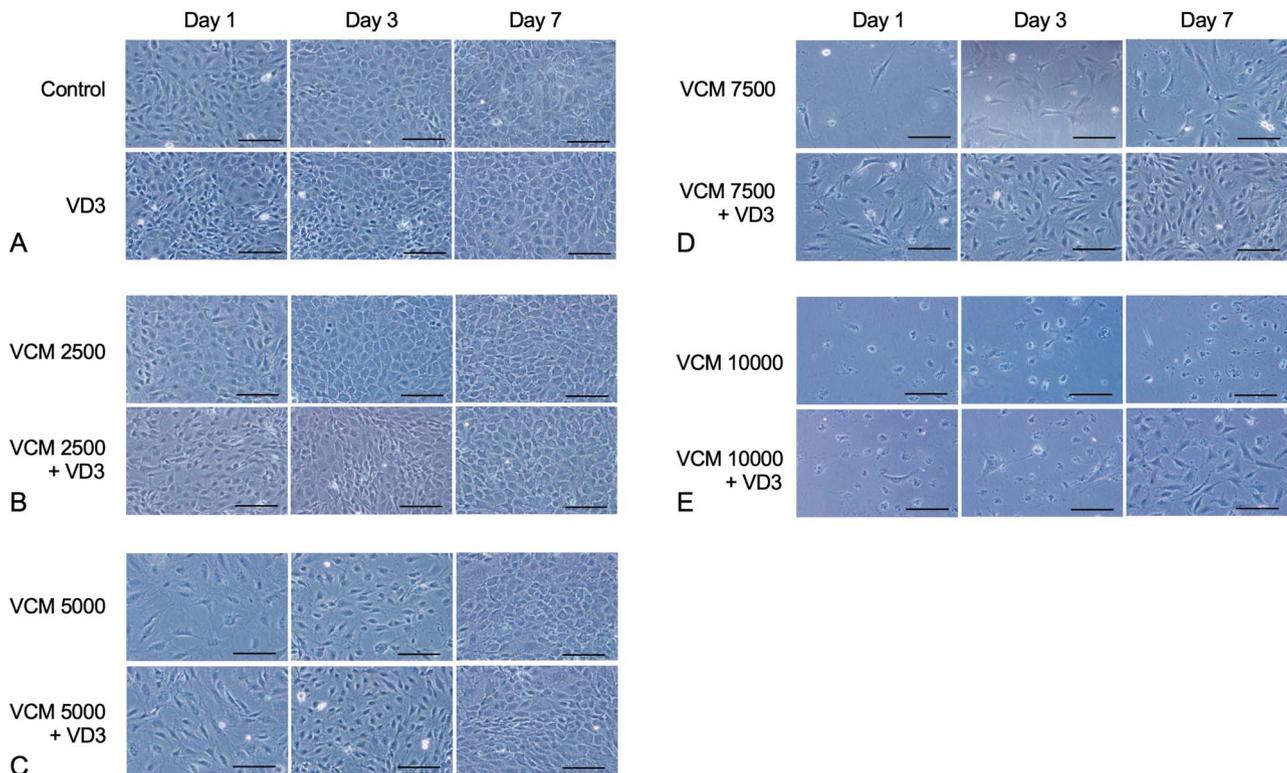
When osteoblasts were treated with vancomycin via pulsed exposure for the first 24 hours in the presence of vitamin D3, we observed an increased number of cells at all examined timepoints (Fig. 7A-C). Vitamin D3 had a recovery effect when vancomycin (2500  $\mu$ g/mL) was administered only for 24 hours (cell number with 2500  $\mu$ g/mL, day 7: vancomycin alone  $15.0 \times 10^4$  cells/mL  $\pm 1.03 \times 10^4$  cells/mL, vancomycin + vitamin D3  $21.2 \times 10^4$  cells/mL  $\pm 0.490 \times 10^4$  cells/mL, mean difference  $6.17 \times 10^4$  cells/mL  $\pm 1.14 \times 10^4$  cells/mL [95% CI 3.80 to 8.53];  $p < 0.001$ ). Vitamin D3 had a recovery effect when vancomycin (5000  $\mu$ g/mL) was administered only for 24 hours (cell number with 5000  $\mu$ g/mL, day 7: vancomycin alone  $13.8 \times 10^4$  cells/mL  $\pm 0.601 \times 10^4$  cells/mL, vancomycin + vitamin D3  $16.4 \times 10^4$  cells/mL  $\pm 0.499 \times 10^4$  cells/mL, mean



**Fig. 7A-C** This figure shows the effect of vitamin D3 in the presence of vancomycin on the number of MC3T3-E1 osteoblast cells 24 hours after administration. Cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL. The cells were exposed to various concentrations of vancomycin and 0.01 nM of vitamin D3. After 24 hours, vancomycin was removed. Cells in the vancomycin-only groups were cultured in an  $\alpha$ -minimum essential medium supplemented with 5% fetal bovine serum, with the addition of 0.01 nM of vitamin D3 in cells in the vitamin D3 groups for (A) 1 day, (B) 3 days, and (C) 7 days. Error bars show the mean  $\pm$  SD. The data represent three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; VCM = vancomycin; VD3 = vitamin D3.

difference  $2.58 \times 10^4$  cells/mL  $\pm$   $0.781 \times 10^4$  cells/mL [95% CI 0.963 to 4.20];  $p < 0.01$ ). Vitamin D3 had a recovery effect when vancomycin (7500  $\mu$ g/mL) was administered only for 24 hours (cell number with 7500  $\mu$ g/mL, day 7: vancomycin alone  $3.46 \times 10^4$  cells/mL  $\pm$   $0.292 \times 10^4$  cells/mL, vancomycin + vitamin D3  $10.6 \times 10^4$

cells/mL  $\pm$   $0.900 \times 10^4$  cells/mL, mean difference  $7.13 \times 10^4$  cells/mL  $\pm$   $0.946 \times 10^4$  cells/mL [95% CI 5.16 to 9.09];  $p < 0.001$ ). The microscopic examination revealed vitamin D3 had a protective effect on osteoblast cells exposed to a high concentration of vancomycin (7500  $\mu$ g/mL) (Fig. 8A-E). Compared with cells in the 7500  $\mu$ g/mL vancomycin



**Fig. 8A-E** This figure shows the protective effects of vitamin D3 on the morphology of MC3T3-E1 osteoblast cells after the administration of vancomycin for 24 hours. Cells were added to a flask at a density of  $1.0 \times 10^4$  cells/mL. The cells were exposed to (A) 0  $\mu\text{g/mL}$ , (B) 2500  $\mu\text{g/mL}$ , (C) 5000  $\mu\text{g/mL}$ , (D) 7500  $\mu\text{g/mL}$ , and (E) 10000  $\mu\text{g/mL}$  of vancomycin and 0.01 nM of vitamin D3 for 24 hours. Thereafter, vancomycin was removed, and cells in the vancomycin-only groups were cultured in an  $\alpha$ -minimum essential medium supplemented with 5% fetal bovine serum. Cells in the vancomycin + vitamin D3 group were cultured in the same medium, with the continued addition of 0.01 nM of vitamin D3. Cells were cultured for 1 day, 3 days, and 7 days. Cell proliferation and morphologic changes were examined using a light microscope. The data represent three independent experiments. Scale bars represent 50  $\mu\text{m}$ ; VCM = vancomycin; VD3 = vitamin D3.

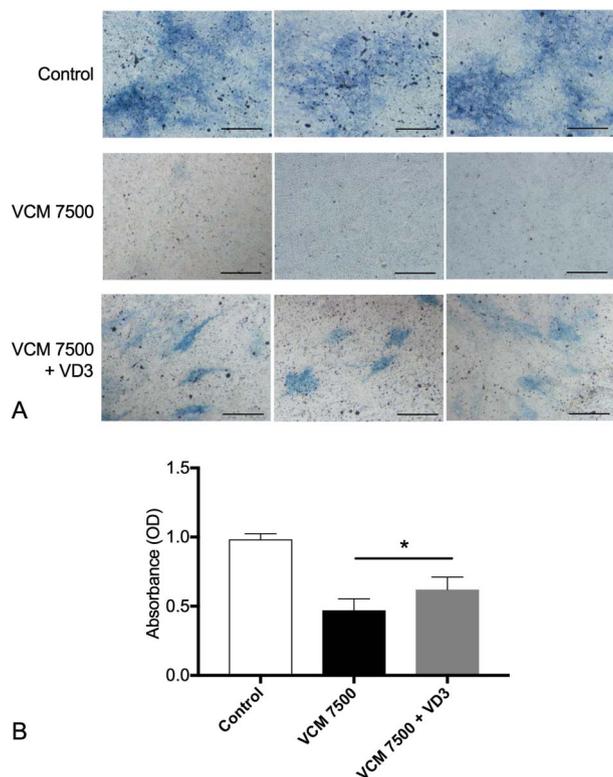
group, those in the 7500  $\mu\text{g/mL}$  vancomycin + 0.01 nM vitamin D3 group tended to develop spherical morphology, and the number of cells increased in a time-dependent manner. The observed morphological changes indicate that vitamin D3 also prevented damage to cells in response to treatment with a higher concentration of vancomycin (10,000  $\mu\text{g/mL}$ ).

#### Vitamin D3 Facilitates Normal Differentiation, Calcification and Gene Expression in Osteoblasts Treated with Vancomycin

Alkaline phosphatase activity in untreated cells was clearly observed under the microscope, whereas very little staining was observed in osteoblast cells after exposure to vancomycin (Fig. 9A). However, we also observed recovery of alkaline phosphatase staining after the addition of vitamin D3 (Fig. 9A). Similar differences were observed using an alkaline phosphatase activity kit (Fig. 9B). There was recovery of

alkaline phosphatase staining upon addition of vitamin D3; this is evidence that vitamin D3 helps restore differentiation. Calcification of untreated osteoblast cells was clearly observed under the microscope, whereas very little staining was observed in osteoblast cells after exposure to vancomycin (Fig. 10). However, recovery of staining, indicating calcification, was observed after the addition of vitamin D3 (Fig. 10).

With the addition of vitamin D3, we observed recovery of alkaline phosphatase staining and Alizarin red staining (evidence of calcification) but no difference in the gene expression of Type I collagen (vancomycin alone  $0.319 \pm 0.0730$ , vancomycin + vitamin D3  $0.511 \pm 0.139$ , mean difference  $0.192 \pm 0.157$  [95% CI -0.483 to 0.867];  $p = 0.345$ ), alkaline phosphatase (vancomycin alone  $0.532 \pm 0.0210$ , vancomycin + vitamin D3  $0.785 \pm 0.0590$ , mean difference  $0.253 \pm 0.0620$  [95% CI -0.015 to 0.521];  $p = 0.055$ ), and cathelicidin antimicrobial peptide (vancomycin alone  $0.885 \pm 0.0520$ , vancomycin + vitamin D3  $1.24 \pm 0.125$ , mean difference  $0.355 \pm 0.135$  [95% CI -0.020 to 0.730];  $p = 0.0580$ ) (Fig. 11).



**Fig. 9A-B** This figure shows the assessment of alkaline phosphatase staining and alkaline phosphatase activity in the 7500  $\mu\text{g}/\text{mL}$  vancomycin and 7500  $\mu\text{g}/\text{mL}$  vancomycin + 0.01 nM vitamin D3 groups. Alkaline phosphatase staining: MC3T3-E1 cells were added to the wells of a 24-well plate at a density of  $1.0 \times 10^4$  cells/mL. After 7 days, alkaline phosphatase staining was performed. Cells were observed (**A**) microscopically. Scale bars represent 50  $\mu\text{m}$ . Alkaline phosphatase activity: MC3T3-E1 cells were added to the wells of a 96-well plate at a density of  $1.0 \times 10^4$  cells/mL. (**B**) After 7 days, alkaline phosphatase activity was measured using an alkaline phosphatase kit. Error bars show the mean  $\pm$  SD. The data represent three independent experiments.  $n = 6$ ;  $*p < 0.05$ ; VCM = vancomycin; VD3 = vitamin D3.

## Discussion

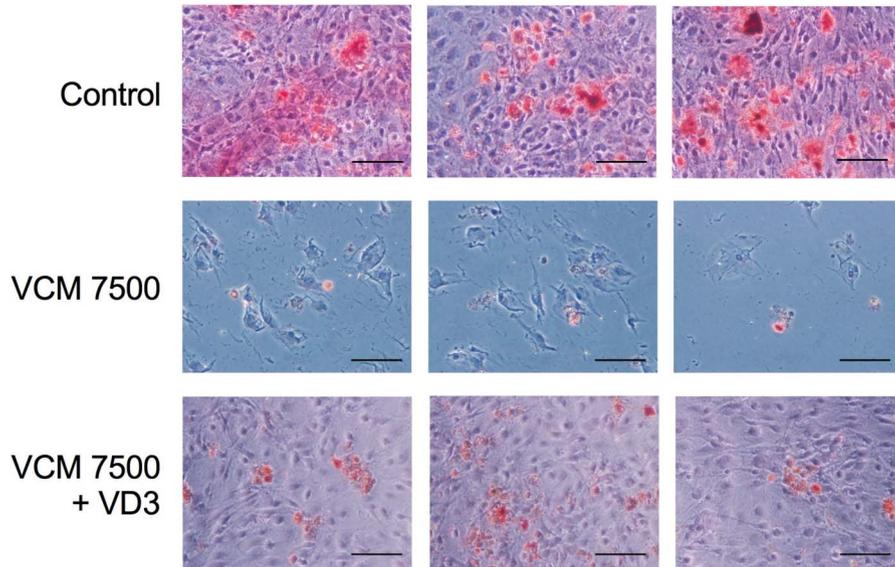
The utility of vancomycin powder in spinal surgery has been studied extensively and is considered a standard for the care and prevention of surgical site infections [7, 8, 14, 23, 24]. However, little is known about the potential adverse effects, such as the inhibition of osteogenesis after vancomycin treatment. The use of concomitantly administered drugs that promote osteogenesis could be one strategy to ameliorate high-dose vancomycin-induced cytotoxicity in osteoblasts. In this study, we investigated vancomycin-induced cytotoxicity in osteoblasts in vitro. Moreover, we concluded that vitamin D3 is a candidate concomitant drug that facilitates the recovery of osteoblasts from vancomycin-induced cytotoxicity.

In this respect, it is also necessary to consider the optimal method for co-administering vancomycin and vitamin D3 to a surgical site. Furthermore, a notable limitation of the present study is that we only investigated the effect of a single dose level of vitamin D3 using a 0.01 nM concentration of vitamin D3, as in another study [12]. However, in preliminary experiments, we examined the effects of this vitamin on cell numbers and morphology at concentrations ranging from 0.01 nM to 100 nM, which indicated that vitamin D3 is not cytotoxic at concentrations under 1 nM (data not shown). Studies regarding the optimal concentration of vitamin D3 in this context are currently ongoing.

The amount of vancomycin used empirically generally depends on the area of a given surgical wound [1]. However, it has been reported that vancomycin doses of 0.5 g to 6 g are the most frequently used [8, 18]. Apart from wound size, the criteria for determining the amount of vancomycin to use clinically remain poorly defined. High concentrations of topically applied vancomycin may cause cytotoxicity in osteoblasts [5]. That report investigated the migration and diffusion of vancomycin and the viability of osteoblasts collected from 10 patients after vancomycin administration. Furthermore, Rathbone et al. [21] reported that vancomycin at a concentration of 5000  $\mu\text{g}/\text{mL}$  is toxic and inhibits the differentiation of osteoblasts. We found that vancomycin at 2500  $\mu\text{g}/\text{mL}$  to 5000  $\mu\text{g}/\text{mL}$  was cytotoxic for osteoblasts, albeit for a short time, whereas at 7500  $\mu\text{g}/\text{mL}$ , vancomycin was conspicuously cytotoxic for a longer period. In that report, vancomycin at concentrations greater than 5000  $\mu\text{g}/\text{mL}$  induced osteoblast cytotoxicity at early timepoints after continuous exposure to vancomycin. Our in vitro data indicate that osteoblast cells are potentially damaged by long-term application of 3 g of vancomycin to wounds during postoperative care.

Vitamin D3 is frequently used to treat osteoporosis and not only suppresses fractures but also enhances calcium absorption from the gastrointestinal tract and modulates parathyroid hormone levels [21]. Clinically,  $1\alpha$ , 25-dihydroxy vitamin D3 (calcitriol) is administered orally, in response to which vertebral body and femoral-fracture suppression effects have been reported [25, 26]. Vitamin D3 has a number of effects on osteoblasts including the induction of proliferation, differentiation, and mineralization [27]. Moreover, vitamin D3 promotes the production of antimicrobial peptides in host cells, inhibits the production of inflammatory cytokines, and stimulates innate immune responses [16]. These host responses induced by vitamin D3 could be beneficial to treat infectious disease. In the present study, we failed to detect an effect of vitamin D3 when co-administered with vancomycin under conditions in which osteoblasts were continuously exposed to vancomycin.

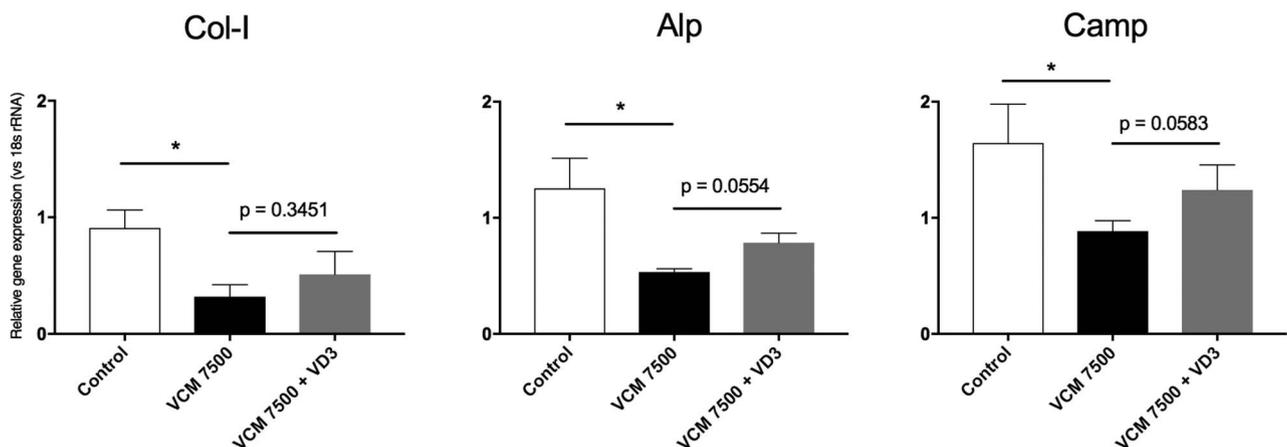
Given that, in clinical practice, vancomycin is removed from wounds using a drain by internal effusion, vancomycin



**Fig. 10** This figure shows our analysis of calcified nodules in the 7500  $\mu\text{g}/\text{mL}$  vancomycin and 7500  $\mu\text{g}/\text{mL}$  vancomycin + 0.01 nM vitamin D3 groups. MC3T3-E1 cells were added to the wells of a 12-well plate at a density of  $1.0 \times 10^4$  cells/mL. After 28 days, Alizarin red S staining was performed to evaluate the mineralization level of MC3T3-E1 cells. Cells were observed microscopically. Scale bars represent 50  $\mu\text{m}$ ; VCM = vancomycin; VD3 = vitamin D3.

concentrations tend to fluctuate at early timepoints. Generally, drain tubes are removed 24 hours postoperatively. To ensure our experiments closely simulated conditions seen in clinical practice, we used an exposure time of 24 hours. We found effects of combined vitamin D3 and vancomycin, resulting in recovery of osteoblast cell health when exposed simultaneously to both substances before the removal of vancomycin after 24 hours (as used in clinical practice).

In these conditions, we observed that alkaline phosphatase activity and the mineralization of osteoblast cells were substantially increased. These results indicate that a combined effect could depress vancomycin-induced cytotoxicity in osteoblasts. Additionally, vitamin D3 stimulates the production of antimicrobial peptides in a wide variety of human cell types [2]. However, with the addition of vitamin D3, we had no difference in the cathelicidin antimicrobial peptide.



**Fig. 11** Reverse transcription-polymerase chain reaction analysis of osteogenesis-related mRNA expression was performed in the 7500  $\mu\text{g}/\text{mL}$  vancomycin and 7500  $\mu\text{g}/\text{mL}$  vancomycin + 0.01 nM vitamin D3 groups. MC3T3-E1 cells were added to the wells of a 96-well plate at a density of  $1.0 \times 10^4$  cells/mL. We assessed the expression of Type I collagen, alkaline phosphatase, and cathelicidin antimicrobial peptide, which are expressed during osteoblast differentiation, using reverse transcription-polymerase chain reaction. Error bars show the mean  $\pm$  SD. The data represent three independent experiments.  $n = 3$ ; \* $p < 0.05$ ; VCM = vancomycin; VD3 = vitamin D3; Col- I = Type I collagen; Alp = alkaline phosphatase; Camp = cathelicidin antimicrobial peptide.

Our results indicate that vitamin D3 may be useful not only in preventing vancomycin-induced cytotoxicity but also for maintaining infection prophylaxis by antimicrobial peptides produced when vancomycin concentrations are decreased.

In summary, we found vancomycin administered as a postoperative antibacterial drug is cytotoxic to osteoblasts at the concentrations used most frequently. We found that vitamin D3 administered in combination with vancomycin can reduce cytotoxicity caused by this antibiotic in the powder topical setting for 24 hours. However, further investigations are necessary to assess whether these findings could be applied in clinical practice in the future.

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