

# Effects of high antibiotic concentrations applied to continuous local antibiotic perfusion on human bone tissue-derived cells

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## Aims

Continuous local antibiotic perfusion (CLAP) has recently attracted attention as a new drug delivery system for orthopaedic infections. CLAP is a direct continuous infusion of high-concentration gentamicin (1,200 µg/ml) into the bone marrow. As it is a new system, its influence on the bone marrow is unknown. This study aimed to examine the effects of high-concentration antibiotics on human bone tissue-derived cells.

## Methods

Cells were isolated from the bone tissue grafts collected from six patients using the Reamer-Irrigator-Aspirator system, and exposed to different gentamicin concentrations. Live cells rate, apoptosis rate, alkaline phosphatase (ALP) activity, expression of osteoblast-related genes, mineralization potential, and restoration of cell viability and ALP activity were examined by in vitro studies.

## Results

The live cells rate (the ratio of total number of cells in the well plate to the absorbance-measured number of live cells) was significantly decreased at ≥ 500 µg/ml of gentamicin on day 14; apoptosis rate was significantly increased at ≥ 750 µg/ml, and ALP activity was significantly decreased at ≥ 750 µg/ml. Real-time reverse transcription-polymerase chain reaction results showed no significant decrease in the ALP and activating transcription factor 4 transcript levels at ≥ 1,000 µg/ml on day 7. Mineralization potential was significantly decreased at all concentrations. Restoration of cell viability was significantly decreased at 750 and 1,000 µg/ml on day 21 and at 500 µg/ml on day 28, and ALP activity was significantly decreased at 500 µg/ml on day 28.

## Conclusion

Our findings suggest that the exposure concentration and duration of antibiotic administration during CLAP could affect cell functions. However, further in vivo studies are needed to determine the optimal dose in a clinical setting.

## Article focus

- This study examined the effects of high-concentration antibiotics used in continuous local antibiotic perfusion (CLAP) including intramedullary

antibiotic perfusion on human bone tissue-derived cells.

## Key messages

- Live cells rate was maintained until day 7 even at a gentamicin (GM) concentration of 1,000 µg/ml, but decreased on day 14 at  $\geq 500$  µg/ml.
- Alkaline phosphatase activity showed a tendency to decrease with increasing GM concentration, and was significantly lower on day 14 than on day 7 at concentrations of  $\geq 750$  µg/ml.
- Current antibiotic concentrations in CLAP may be sufficient to treat bone and soft-tissue infections, although they could have negative effects on human bone tissue-derived cells.

## Strengths and limitations

- Using the Reamer-Irrigator-Aspirator system, this study investigated the human bone tissue-derived cells that mimic the intra-medullary tissue environment.
- Moreover, GM concentrations that sufficiently covered the concentrations realized in the local environment by CLAP were evaluated.
- This study conducted only an in vitro study, which could be a limitation, and an in vivo examination would be necessary as the next step.

## Introduction

Bone and soft-tissue infections are often difficult to treat, especially fracture-related infections (FRIs), which can be especially challenging owing to the presence of implants. In recent years, despite diagnostic and treatment algorithms for FRIs,<sup>1</sup> the treatment success rate is not as high, and there are still many problems.

Persistent FRIs require longer treatment time, and sometimes multiple surgeries.<sup>2,3</sup> The reasons why FRIs are refractory include increased sequestrum, decreased local blood flow, and biofilm formation.<sup>4,5</sup> A biofilm is a bacterial aggregate embedded in the cellular matrix produced by colonizing bacteria, particularly on implant surfaces.<sup>6,7</sup> The antibiotic concentration that can suppress biofilms is defined as the minimal biofilm eradication concentration (MBEC), which is 100 to 1,000 times the minimal inhibitory concentration (MIC) which, in turn, is the lowest antibiotic concentration that inhibits bacterial growth.<sup>7</sup> However, the occurrence of systemic adverse events makes it difficult to achieve MBEC-exceeding antibiotic concentrations in a local lesion by intravenous antibiotic administration. Moreover, despite the availability of several local antibiotic therapies for FRIs,<sup>8</sup> the local concentration of the antibiotics sustainably released in these therapies does not reach the MBEC.<sup>9,10</sup> Presently, continuous local antibiotic perfusion (CLAP) is attracting attention as a novel drug delivery system that overcomes this problem and enables local antibiotics concentration to exceed the MBEC.<sup>11–13</sup> The CLAP involves local administration and perfusion of high-concentration antibiotics, allowing local antibiotic concentrations to exceed the MBEC safely. In addition, the method is different from systemic administration of antibiotics, as the locally perfused antibiotics are collected using negative wound pressure therapy or a drain tube.

Monitoring the blood concentration of antibiotics is necessary during CLAP. The ideal antibiotics for this therapy are bactericidal, concentration-dependent agents, which

include aminoglycosides,<sup>14,15</sup> among which gentamicin (GM) is used as a standard protocol in a clinical setting.

The standard CLAP protocol, including intramedullary antibiotic perfusion (iMAP), is the continuous administration of 1,200 µg/ml of GM at 2 ml/hr.<sup>11–13</sup> This therapy is usually performed for approximately two weeks. By maintaining a high concentration of antibiotics for the required period, it is possible, even in the late phase of FRIs, to preserve the implant, control the infection, and lead to bone union with fewer complications.<sup>13</sup> However, despite favourable clinical outcomes, the appropriate GM concentration in CLAP and the effects of high-concentration antibiotics on the local cells are unknown. The purpose of this study was to examine how high-concentration antibiotics affected cells.

## Methods

### Patients

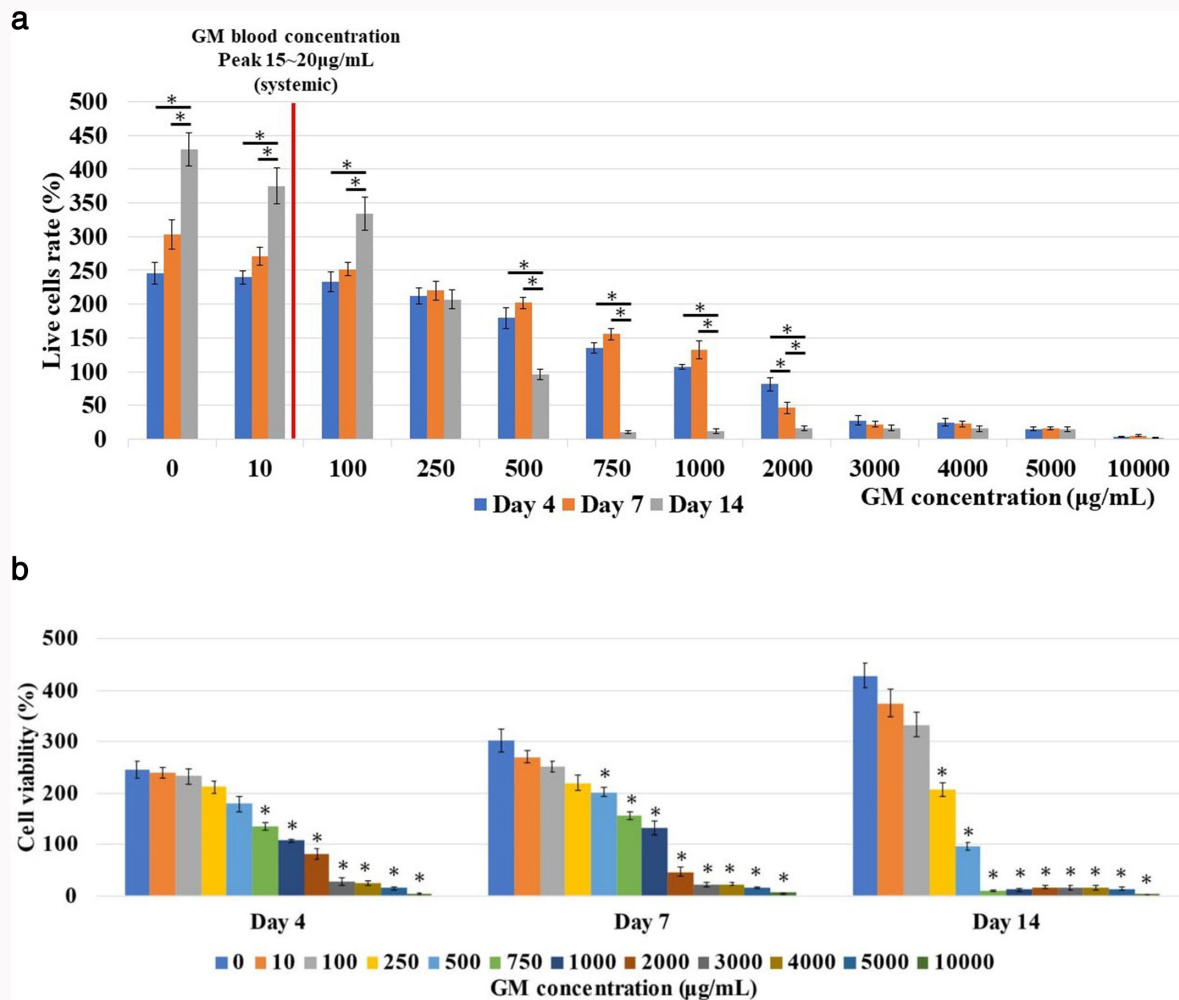
Six patients, including three men and three women, with a mean age of 65.8 years (61 to 71), who underwent treatment for infected nonunion and osteomyelitis between April and September 2020 were included. The cells used in this study were harvested using the Reamer-Irrigator-Aspirator (RIA) system from the femur in each patient. The RIA system (DePuy Synthes, USA), which was originally designed for irrigating the medullary canal of long bones and is currently used to harvest autologous bone grafts by excavating the medullary canal, was used to harvest the patients' bone tissues (Supplementary Table i).<sup>16</sup> The institutional review board of our hospital approved this study, and written informed consent was obtained from all patients (Project ID 180067).

### Isolation and culture of human bone tissue-derived cells

A small part of the RIA graft was obtained for this study. Since the RIA graft tissue is reported to contain a large number of mesenchymal stem cells and heterogeneous cell populations,<sup>17–19</sup> we believe that cells collected from the RIA system in this study are suitable to investigate a situation mimicking the real intra-bone marrow environment.

The RIA grafts were minced into small pieces in the modified minimum essential medium (Sigma-Aldrich, USA) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco, USA), and antibiotics. The cultures were incubated at 37°C under 5% humidified CO<sub>2</sub>. Approximately two weeks later, the adherent cells were harvested with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (Wako, Japan) and passaged. Cells at passage 2 or 3 were used in the following assays.

The cells were seeded into well-plates according to each assay described below. Before use, cells were incubated in a growth medium at 37°C under 5% humidified CO<sub>2</sub> for three days. Assays for osteogenic potential were performed using the osteogenic medium, which is growth medium supplemented with 10 nM dexamethasone (Sigma), 10 mol/l β-glycerophosphate (Sigma-Aldrich), and 50 mg/ml ascorbic acid (Wako). Cell viability (except for restoration assay) was performed using 12 different concentrations of gentamicin sulfate (GM) (Wako) in the range of 0 to 10,000 µg/ml, and the other assays were performed using seven different GM concentrations in the range of 0 to 2,000 µg/ml.



**Fig. 1**

Analysis of live cells rate, which was calculated by setting the rate at the start of exposure as 100%. a) Comparison at each gentamicin (GM) concentration. For reference, the peak blood concentration of 15 to 20 µg/ml after regular systemic administration of gentamicin is shown as a red line in the figure. b) Analysis of cell viability. Each concentration is compared to 0 µg/ml. \* $p < 0.05$  (Kruskal-Wallis test and Steel test).

### Live cells rate (cell viability)

Live cells rate was assessed as described in the Supplementary Methods section. In addition, restoration of cell viability after GM exposure was also examined. After the culture in GM for one to two weeks, the cells were cultured in a growth medium for an additional two weeks without GM. The absorbance values at the end of GM exposure and the end of the total culture were evaluated.

### Apoptosis rate assay

Apoptosis rate was determined as described in the Supplementary Methods section. This assay was performed 48 hours after GM exposure.

### Alkaline phosphatase activity assay

Cells that were collected and suspended in phosphate-buffered saline on days 7 and 14 were sonicated using a Microson Ultrasonic Cell Disruptor XL2000 (Misonix, USA) and stored at  $-20^{\circ}\text{C}$  until use. Alkaline phosphatase (ALP) activity was assessed as described in the Supplementary methods section.

Restoration of ALP activity was also examined. After GM exposure for two weeks, the cells were subsequently cultured in an osteogenic medium without GM for two weeks.

Absorbance values at 405 nm were measured at the end of GM exposure and the end of the total culture.

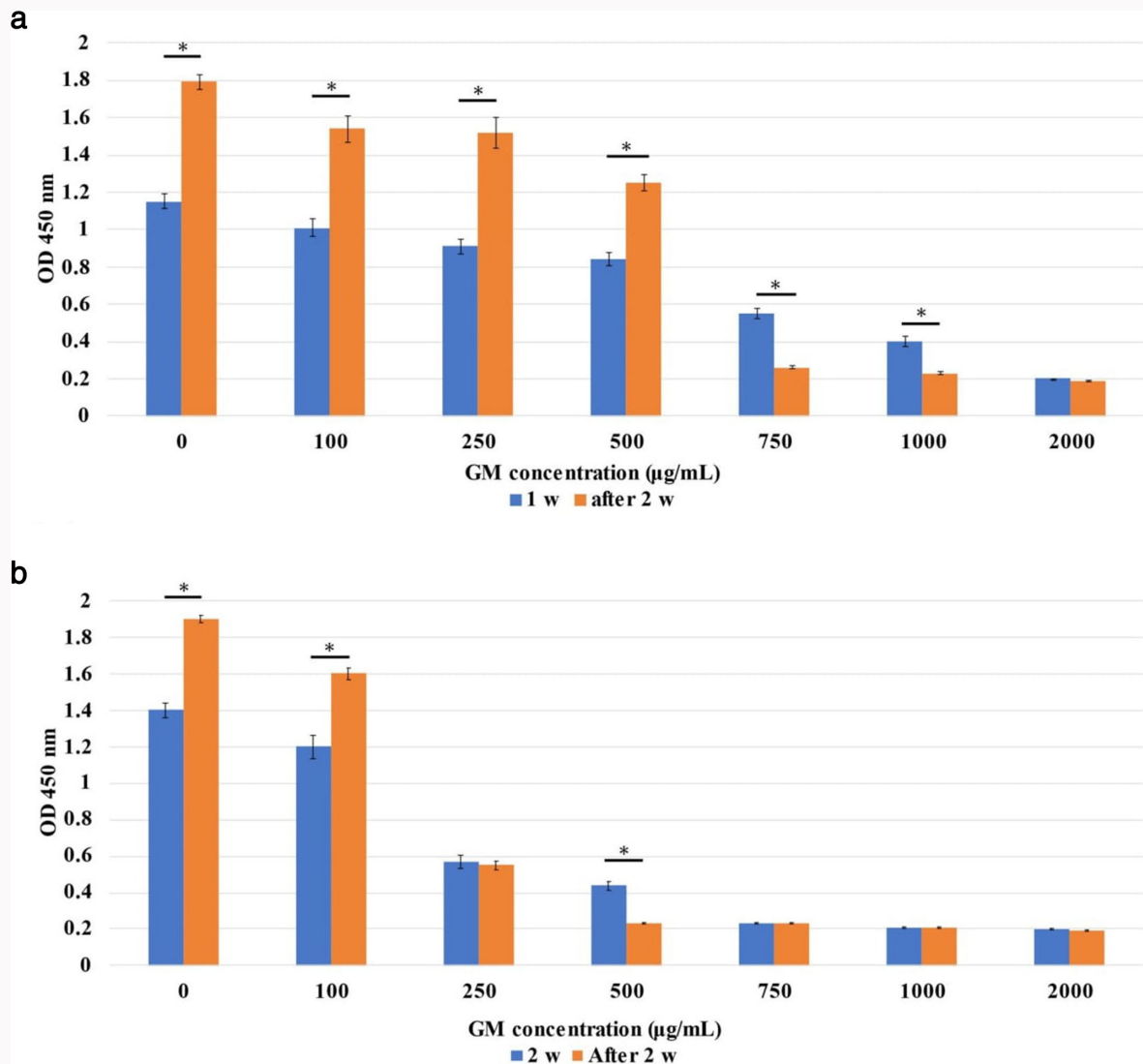
### Real-time reverse transcription-polymerase chain reaction

The total RNA of the cultured cells was subjected to reverse transcription-polymerase chain reaction (RT-PCR) to determine the expression levels of genes as described in the Supplementary methods section. The primers used are listed in Supplementary Table ii.

Real-time RT-PCR was performed using a thermal cycler (Programme Temperature Control System PC-707; ASTEC; Japan). The expression level of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene, and was presented as the fold change relative to the data of day 0 using the  $\Delta\Delta\text{CT}$  method (Applied Biosystems; Thermo Fisher Scientific, USA).<sup>20</sup>

### Mineralization assay

Mineralization assay to evaluate the calcification of the extracellular matrix during osteogenic differentiation is described in detail in the Supplementary methods section.



**Fig. 2**

Analysis of restoration of cell viability, with absorbance comparison at each timepoint. a) Comparison of absorbance over a total of three weeks at each gentamicin (GM) concentration (after one week of exposure, followed by culturing for two weeks without GM). b) Comparison of absorbance over a total of four weeks at each GM concentration (after two weeks of exposure, followed by culturing for two weeks without GM). \* $p < 0.05$  (Mann-Whitney U test). OD, optical density.

### Statistical analysis

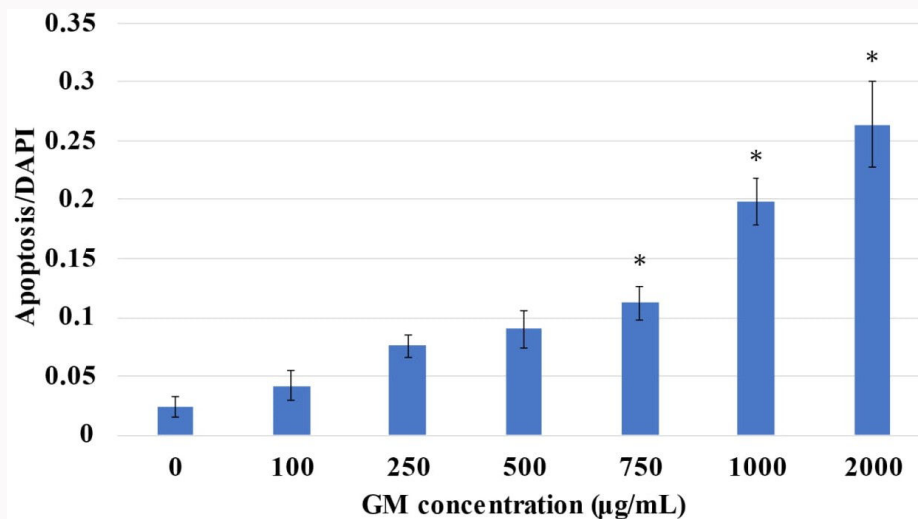
All data are expressed as the mean and standard error (SE). All statistical analyses were performed with EZR (Saitama Medical Centre, Jichi Medical University, Japan),<sup>21</sup> which is a graphical user interface for R (R Foundation for Statistical Computing, Austria). The Kruskal-Wallis test and Steel test were used to compare differences in cell viability, apoptosis rate, gene expression levels, and mineralization at each GM concentration at different timepoints. To determine significant differences in ALP activity, and restoration of cell viability and ALP activity between the timepoints, we performed the Mann-Whitney U test. Differences were considered statistically significant at  $p < 0.05$ .

### Results

#### Live cells rate (cell viability)

Up to 100 µg/ml GM, the difference in cell viability between days 4 and 7 was insignificant, but was significantly higher on day 14 than on days 4 and 7 (0 µg/ml:  $p = 0.006$  for day 4 vs

day 14,  $p = 0.008$  for day 7 vs day 14, 10 µg/ml:  $p = 0.006$  for day 4 vs day 14,  $p = 0.030$  for day 7 vs day 14, 100 µg/ml:  $p = 0.006$  for day 4 vs day 14,  $p = 0.030$  for day 7 vs day 14; Kruskal-Wallis test and Steel test). At 250 µg/ml, no significant difference was observed between the different timepoints. At 500, 750, 1,000, and 2,000 µg/ml GM, cell viability was significantly lower on day 14 than on days 4 and 7 (500 µg/ml:  $p = 0.006$  for day 4 vs day 14,  $p = 0.006$  for day 7 vs day 14, 750 µg/ml:  $p = 0.006$  for day 4 vs day 14,  $p = 0.006$  for day 7 vs day 14, 1,000 µg/ml:  $p = 0.006$  for day 4 vs day 14,  $p = 0.006$  for day 7 vs day 14, 2,000 µg/ml:  $p = 0.006$  for day 4 vs day 14,  $p = 0.017$  for day 7 vs day 14, Kruskal-Wallis test and Steel test). At 2,000 µg/ml, cell viability was significantly lower on day 7 than on day 4 ( $p = 0.041$ , Kruskal-Wallis test and Steel test), and at  $\geq 3,000$  µg/ml it tended to decrease at all timepoints, although with insignificant difference between different time points (Figure 1a). For reference, the peak blood concentration of 15 to 20 µg/ml after regular systemic administration of gentamicin is shown as a red line in the figure. Regard-



**Fig. 3**

Apoptosis rate. The ratio of green-stained nuclear fragments to 4',6-diamidino-2-phenylindole (DAPI)-stained cells was calculated for each of the four fields of view after 48 hours of gentamicin (GM) exposure. Each concentration is compared to 0 µg/ml. \* $p < 0.05$  (Kruskal-Wallis test and Steel test).

ing different timepoints, cell viability was significantly lower at  $\geq 750$ ,  $\geq 500$ , and  $\geq 250$  µg/ml GM on days 4, 7, and 14, respectively, than at 0 µg/ml (day 4:  $p = 0.033$ , day 7:  $p = 0.033$ , day 14: 0.033, Kruskal-Wallis test and Steel test) (Figure 1b).

Regarding restoration of cell viability, cell viability was significantly higher in the three-week culture than in the one-week culture with GM at  $\leq 500$  µg/ml and significantly decreased with GM at 750 and 1,000 µg/ml (Figure 2a). Cell viability was significantly higher in the four-week culture than in the two-week culture, with GM up to 100 µg/ml. However, no significant difference was observed with GM at 250 µg/ml and  $\geq 750$  µg/ml, whereas the activity was significantly decreased with GM at 500 µg/ml (Figure 2b).

### Apoptosis rate

The apoptosis rate was semi-quantified as the ratio of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-stained nuclear fragmentation (green) to 4',6-diamidino-2-phenylindole (DAPI)-stained cells (blue). The images at 100 and 1,000 µg/ml GM are shown as representative images (Supplementary Figure a). The apoptosis rate was significantly higher at  $\geq 750$  µg/ml than at 0 µg/ml GM (750 µg/ml:  $p = 0.031$ , 1,000 µg/ml:  $p = 0.019$ , 2,000 µg/ml:  $p = 0.019$ , Kruskal-Wallis test and Steel test) (Figure 3).

### ALP activity

ALP activity was significantly higher at 100 µg/ml and significantly lower at  $\geq 750$  µg/ml GM on day 14 than on day 7 (100 µg/ml:  $p = 0.002$ , 750 µg/ml:  $p = 0.002$ , 1,000 µg/ml:  $p = 0.015$ , 2,000 µg/ml:  $p = 0.004$ , Mann-Whitney U test). No significant difference was observed between 250 and 500 µg/ml GM (250 µg/ml:  $p = 0.937$ , 500 µg/ml:  $p = 0.699$ , Mann-Whitney U test) (Figure 4a). ALP activity was significantly lower at  $\geq 100$  µg/ml GM on day 7 and  $\geq 250$  µg/ml GM on day 14 than at 0 µg/ml (Figure 4b).

Regarding restoration of ALP activity, no significant difference was observed up to 250 µg/ml GM. At 500 µg/ml, the restorative ability was significantly decreased after a two-week culture without GM. At 750 and 1,000 µg/ml,

the restorative ability remained low, although no significant difference was observed between the two concentrations (750 µg/ml:  $p = 0.818$ , 1,000 µg/ml:  $p = 0.093$ , Mann-Whitney U test). At 2,000 µg/ml, there was a significant difference in the restorative ability, albeit at a low level (Figure 5).

### Real-time RT-PCR

When compared to 0 µg/ml GM, the transcription levels of runt-related transcription factor 2 (RUNX2), osteocalcin (OC), and bone morphogenetic protein 2 (BMP2) at 2,000 µg/ml GM; osterix (OSX) at  $\geq 1,000$  µg/ml; and collagen, type I,  $\alpha$  1 (COL1A1) at  $\geq 250$  µg/ml were significantly decreased on day 7, whereas those of ALP and activating transcription factor 4 (ATF4) did not show any significant difference. Expression levels of RUNX2 at  $\geq 500$  µg/ml GM; OSX, ATF4, and BMP2 at  $\geq 1,000$  µg/ml; ALP and OC at  $\geq 750$  µg/ml; and COL1A1 at  $\geq 100$  µg/ml were significantly decreased on day 14 (Figure 6).

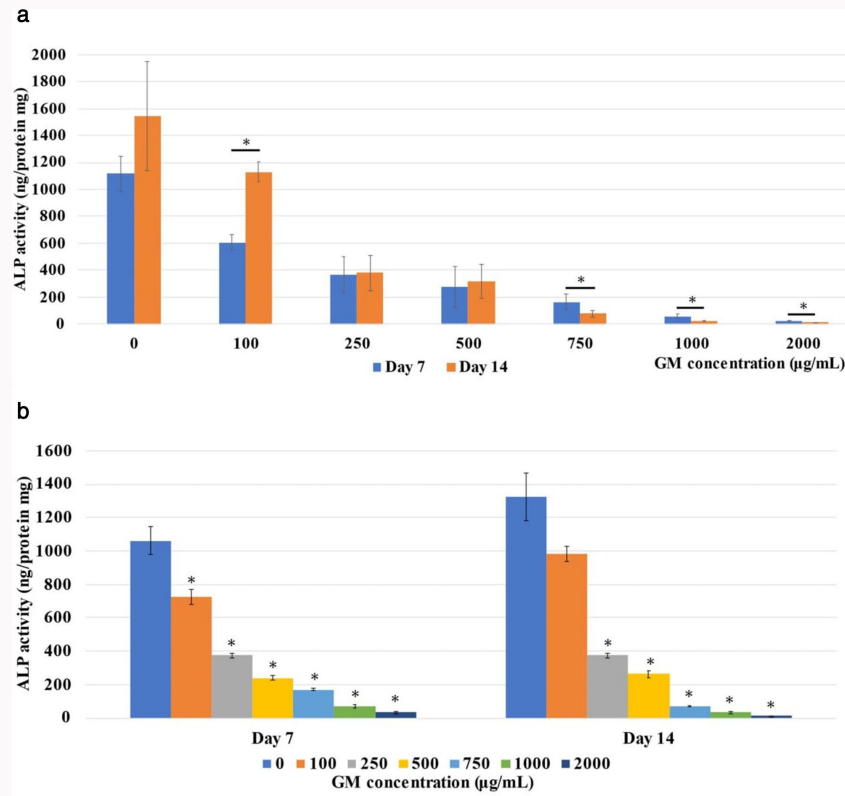
### Mineralization potential

The mineralization potential was observed to reduce with increasing concentration of GM in all samples, as indicated by the calcified areas stained by Alizarin Red S (Supplementary Figure b). Compared to 0 µg/ml GM, the mineralization potential was significantly reduced in all samples at  $\geq 100$  µg/ml; compared to 100 µg/ml GM, the mineralization potential was significantly reduced in all samples at  $\geq 500$  µg/ml; compared to 250 µg/ml all were significantly decreased at  $\geq 750$  µg/ml; and compared to 500 µg/ml, all were significantly decreased at  $\geq 1,000$  µg/ml (Figure 7).

### Discussion

This study found that at GM concentrations of 500 to 1,000 µg/ml, live cells rate was maintained for up to seven days, but significantly decreased at 14 days. In contrast, at a GM concentration of 2,000 µg/ml, live cells rate decreased significantly over time. These results suggest that live cells rate may be suppressed at the clinical dosage of GM (1,200 µg/ml) for 14 days. In contrast to previous studies,<sup>22,23</sup> our study found no significant difference at 100 µg/ml compared to





**Fig. 4**

Analysis of alkaline phosphatase (ALP) activity. a) Comparison at each gentamicin (GM) concentration. \* $p < 0.05$  (Mann-Whitney U test). b) Comparison at each timepoint. \* $p < 0.05$  (Kruskal-Wallis test and Steel test). Each GM concentration was compared with 0 µg/ml.

0 µg/ml. The cells from RIA graft used in this study are reported to proliferate faster<sup>24</sup> and to contain more growth factors<sup>25</sup> than cells from iliac bone, suggesting that the RIA cells have higher proliferative potential. This difference in cell characters between the previous studies and the current study could explain why the effect in low concentration was not apparent in this study. However, we did observe a trend of decreasing live cells rate with increasing antibiotic concentration consistent with previous reports.<sup>22,23</sup>

In addition, the restoration of cell viability was also poor at  $\geq 750$  µg/ml after a one-week exposure and at  $\geq 500$  µg/ml after a two-week exposure. This result suggests that although some cells lose their activity or die depending on the GM concentration, the activity of some cells recovers thereafter, including those that survived. However, above a certain concentration, cell activity further decreases even after exposure has stopped, suggesting that the effects of GM on local cells may remain even after exposure has ended.

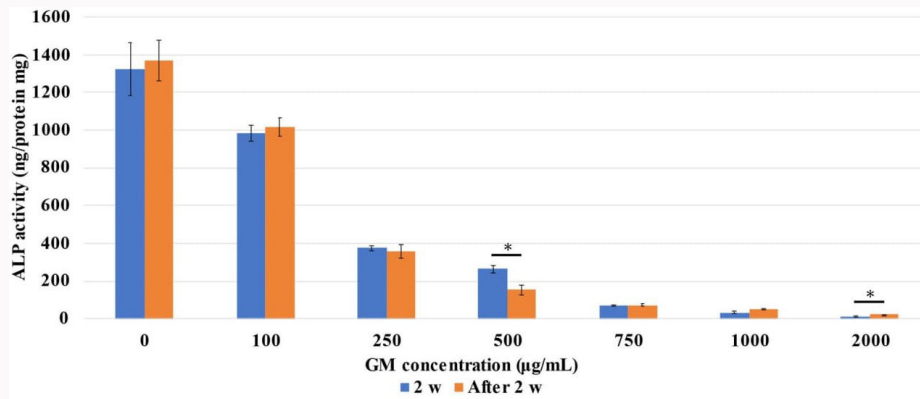
Moreover, the apoptosis rate after 48 hours of exposure tended to increase in a concentration-dependent pattern. Although the timepoint is different from the live cells rate assay, the live cells rate significantly decreased at  $\geq 750$  µg/ml on day 4, which appears to correlate with the apoptosis rate results, indicating that high concentrations of antibiotics may damage cells in the early phase of the treatment.

Based on the results described above, it may be necessary to set the antibiotic concentration lower, as concentrations above a certain level damage cells from an early stage, and the effects remain even after exposure has ended.

Regarding osteogenic potential, both ALP activity, including the restoration of ALP activity, and osteogenic gene expression levels showed a declining trend with increasing GM concentration, similar to that observed for cell viability. In previous reports, ALP activity decreased at GM concentrations of  $\geq 100$  µg/ml,<sup>22</sup> which was observed on day 7 in the current study. ALP activity was significantly lower on day 14 than on day 7 at  $\geq 750$  µg/ml, suggesting that it could decrease over time at the clinical GM concentration in CLAP.

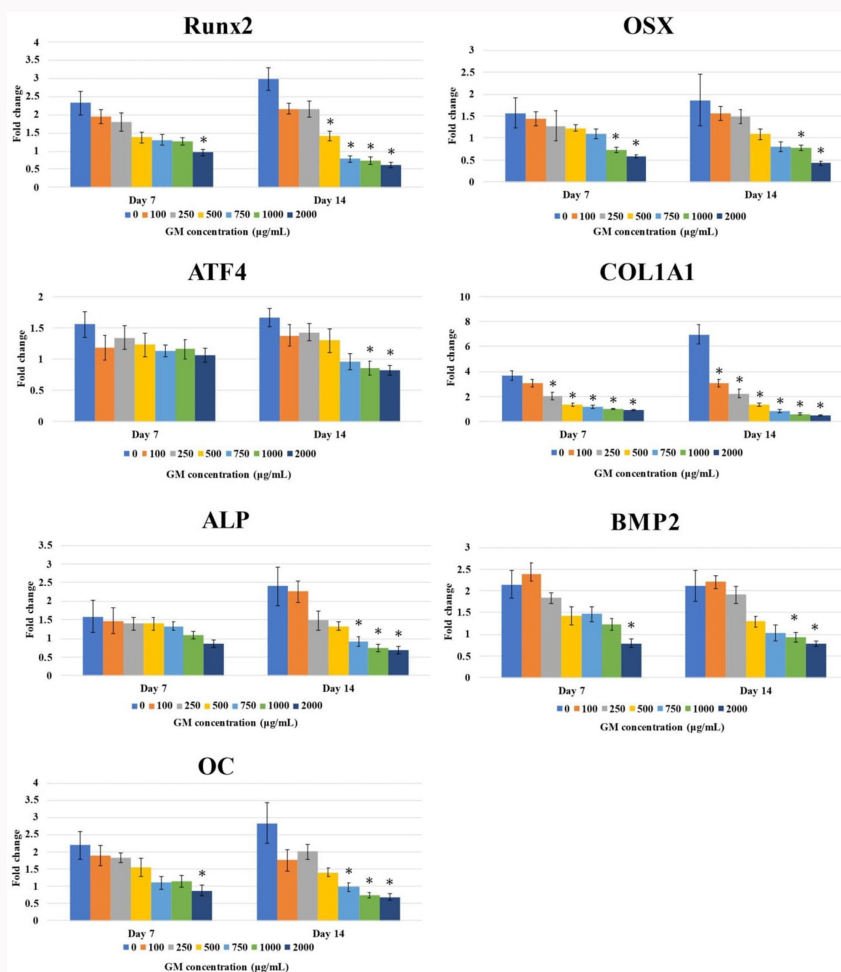
Regarding gene expression, on day 7, compared to that at 0 µg/ml, the expression levels of OSX and COL1A1 were significantly lower at 1,000 µg/ml GM; and ALP and ATF4 were the only exceptions that showed no significant decrease at 2,000 µg/ml GM. However, on day 14, the expression levels of all genes were significantly decreased compared to those at 0 µg/ml. These results suggest that some genes may not be affected by the exposure period up to day 7 with the concentrations examined in this study. ALP and Runx2 are early markers of bone differentiation,<sup>26</sup> whereas OC and ATF4 are considered late markers.<sup>27,28</sup> BMP2 reportedly induces OSX expression.<sup>29</sup> The current results suggest that, up to day 7, even at 1,000 and 2,000 µg/ml GM, the expression of ALP, Runx2, ATF4, BMP2, and OC would remain unchanged and the entire pathway of osteogenic potential may be maintained. However, as the expression levels of all genes were significantly lower on day 14 at both 1,000 and 2,000 µg/ml than at 0 µg/ml, it suggests that a longer exposure may have a greater influence on gene expression.

In clinical practice, the fracture healing rate in FRI with CLAP is 95%.<sup>13</sup> Long-term GM exposure may have a



**Fig. 5**

Analysis of restoration of alkaline phosphatase (ALP) activity. Comparison of ALP activity over a total of four weeks at each gentamicin (GM) concentration (after two weeks of exposure followed by culturing for two weeks without GM). \* $p < 0.05$  (Mann-Whitney U test).

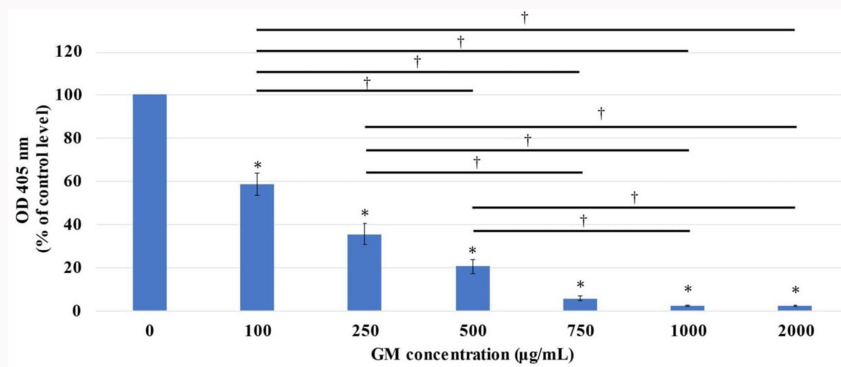


**Fig. 6**

Analysis of the expression of osteoblast-related genes at each gentamicin (GM) concentration. The messenger RNA expression levels of runt-related transcription factor 2 (RUNX2), osterix (OSX), activating transcription factor 4 (ATF4), collagen type I (COL1A1), alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), and osteocalcin (OC) were evaluated and normalized by the mRNA expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). \* $p < 0.05$  (Kruskal-Wallis test and Steel test), each compared to 0 µg/ml of GM.

lower influence on in vivo clinical settings of fracture sites than predicted by the in vitro results. It is possible that the infusion dose of antibiotics from iMAP was diluted by blood or discharge at the bone marrow, and the antibiotic dosage delivered to the local tissue was lower than expected.

The difference in the nourishing tissue environment, such as the surrounding blood flow, between in vivo and in vitro conditions may also be a contributing factor. Thus, the optimal GM concentration in clinical settings remains open to investigation.



**Fig. 7**

Alizarin Red S staining on day 28 (one-week exposure to gentamicin (GM) followed by three weeks' culture without GM). The absorbance at each GM concentration was measured and relative values calculated with 0 µg/ml as 100%. \* $p < 0.05$  (Kruskal-Wallis test and Steel test), each concentration compared to 0 µg/ml of GM; † $p < 0.05$ , comparisons between each concentration. OD, optical density.

The current results suggest that increasing antibiotic concentrations could affect the mineralization potential. In a clinical setting, the bone union is reported to take approximately 7.5 months following CLAP.<sup>13</sup> Considering this report and our results, GM concentrations in CLAP may delay bone union via a reduction in the mineralization potential. Moreover, since no significant difference in mineralization potential was observed among concentrations above 750 µg/ml, it suggests that 750 µg/ml is the threshold for mineralization potential.

Considering the above, the current GM concentrations in CLAP may have a significant effect on cells, although in clinical practice, infection is controlled and bone healing is successfully achieved.

Although treatment at lower antibiotic concentrations should reduce adverse effects, it is essential to know the appropriate antibiotic concentrations that exceed the MBEC, since infection treatment is the main focus of CLAP. A study on staphylococci isolated from foot gangrene reported that the MIC and MBEC of the GM were less than 64 and 256 µg/ml, respectively,<sup>15</sup> suggesting the possibility of administering a lower dosage of GM.

The results of this study suggest that the concentration thresholds for cell proliferation and calcification would be different, and that calcification would be affected even at lower GM concentrations. It would be better if the treatment could be performed at a GM concentration that does not affect calcification. However, if the concentration is lowered to such a level, the GM concentration would likely be < 100 µg/ml, which is lower than the MBEC of the bacteria, and would prevent the original therapeutic objective of calming the infection. As per the definition of the "diamond concept" by Giannodis et al,<sup>30</sup> osteogenic cells are fundamental for bone healing, and it is considered most important that cells with proliferative potential exist locally. In this study, the concentration thresholds for cell proliferative and osteogenic differentiation capacity were generally consistent, suggesting that it may be better to consider antimicrobial concentrations that do not affect cell proliferation in clinical settings.

Although it is difficult to extrapolate the results of this study directly to clinical practice, there is room for further study to determine the optimal antibiotic concentration and whether a pharmacokinetic study in vivo is needed.

We believe the strength of this study is the use of human bone tissue-derived cells from the RIA system, as opposed to previous studies using cell strains. These cells are likely to be affected during CLAP in clinical settings.

This study has some limitations. First, the number of samples was small. By increasing the number of samples, new important findings might be demonstrated, and the interpretation of the results might be different. Second, pharmacokinetics were not considered in the current study because the information regarding pharmacokinetics of GM in vitro is unknown. In addition, although clinical data about pharmacokinetics of GM are of that in general intravenous systemic administration, such pharmacokinetics in CLAP have not been studied. To resolve this limitation, it is first necessary to study pharmacokinetics in a clinical setting. Finally, as this was an in vitro study, the current results regarding the GM concentration cannot be directly extrapolated to clinical practice. The local concentration of antibiotics in CLAP is unknown, and does not reproduce the movement of antibiotics in clinical practice. In this study, GM was added to the culture medium and left to stand. In clinical practice, however, GM is constantly perfusing local areas such as the intrathecal space, where blood and leachate are thought to be present, and it is possible that the local concentration is lower than the administered concentration of the antimicrobial agent. As such, the actual clinical situation was not reproduced, which could have affected the results. For a more detailed evaluation, it will be helpful to evaluate conditions mimicking in vivo situations by fluid mechanics in the future, and to proceed with the investigation of the effects and optimal antibiotic concentration in CLAP.

In conclusion, increased GM concentration affected cell viability, osteogenic potential, and mineralization potential, and some of the GM concentrations used were significantly lower than those used in CLAP. This study can serve as an index for considering the antibiotic concentration; however, further in vivo studies are needed to determine the optimal concentrations for CLAP.

### Supplementary material

Tables showing the patients' clinical data and details of real-time reverse transcription-polymerase chain reaction primers used in this study, along with figures showing fluorescent stained images of



the apoptosis rate assay, representative images of Alizarin Red S staining, and the evaluation methods for each assay.

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