

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

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**INTRODUCTION:** Continuous local antibiotic perfusion (CLAP) has recently attracted attention as a novel drug delivery system for orthopaedic infections [1,2,3]. CLAP includes direct continuous infusion of high-concentration gentamicin (GM) into the bone marrow [1,2,3]. However, its influence on the bone marrow has not been well studied. In this study, we examined the effects of high antibiotic concentrations on human bone tissue-derived cells.

**METHODS:** This study was approved by the medical ethics committee of our hospital.

**Tissue harvesting:** Bone tissue was collected from six patients using a reamer irrigator aspirator (RIA) system, which can harvest bone tissue by reaming the intramedullary canal of the long bones.

**Isolation of human bone tissue-derived cells (HBTDCs):** HBTDCs were isolated as described previously [4]. Briefly, bone tissues collected using RIA were minced into small pieces and grown in modified minimum essential medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco), and various antibiotics.

**Cell viability assay:** A total of  $3 \times 10^3$  HBTDCs per well were seeded in a 96-well plate and incubated for four days. The cells were then divided into 7 groups and exposed to 7 different concentrations of GM from 0 to 2000  $\mu\text{g/mL}$ . Cell viability was determined on days 4, 7, and 14 using the Cell Counting Kit-8 (Dojindo Lab) and compared by taking the value before exposure as 100%. In addition, restoration of cell viability after GM exposure was examined. After culturing in GM for 1–2 weeks, the cells were cultured in a growth medium for an additional 2 weeks without GM. The absorbance values at the end of the GM exposure and at the end of the total culture were evaluated.

**Alkaline phosphatase (ALP) activity assay:** A total of  $4 \times 10^4$  HBTDCs per well were seeded into 6-well plates and incubated in osteogenic medium supplemented with 10 mM-glycerophosphate (Sigma), 50 g/mL ascorbic acid (Wako), and 10 nM dexamethasone (Sigma), for four days. The cells were then exposed to seven different concentrations of GM, as described above, and ALP activity on days 7 and 14 was determined using the SenoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Corporate Headquarters) and calculated by compensating the amount of protein using the Pierce BCA Protein Assay Kit (Thermo Scientific). In addition, restoration of ALP activity was examined. After culturing in GM for 2 weeks, the cells were cultured in osteogenic medium for an additional 2 weeks without GM. ALP activity values at the end of GM exposure and at the end of the total culture were evaluated.

**Mineralization assay:** A total of  $1 \times 10^4$  HBTDCs per well were seeded in a 48-well plate and incubated in osteogenic medium for four days. The cells were cultured in GM-containing medium for 1 week and then in GM-free medium for 3 weeks. Alizarin red S (Iwai Chemicals, Co., Ltd., Tokyo, Japan) concentration was evaluated by measuring the absorbance at 405 nm and expressed as relative intensity levels compared to that at 0  $\mu\text{g/mL}$ .

## RESULTS:

**Cell viability assay.** Cell viability was maintained for 14 days at a GM concentration of 100  $\mu\text{g/mL}$  or less. Meanwhile, exposure to 500–2000  $\mu\text{g/mL}$  of GM significantly decreased between days 7 and 14 (Fig. 1). Restoration of cell viability in the 3-week culture was significantly higher than that in the 1-week culture with GM at  $\leq 500$   $\mu\text{g/mL}$  and significantly decreased with GM at 750 and 1000  $\mu\text{g/mL}$  (Fig. 2a). Cell viability in the 4-week culture was significantly higher than that in the 2-week culture with GM concentrations up to 100  $\mu\text{g/mL}$ . However, no significant difference was observed with GM at 250 and 750  $\mu\text{g/mL}$ , whereas the activity significantly decreased with GM at 500  $\mu\text{g/mL}$  (Fig. 2b).

**ALP activity assay** ALP activity was significantly higher at 100  $\mu\text{g/mL}$  and significantly lower at  $\geq 750$   $\mu\text{g/mL}$  on day 14 than on day 7. No significant differences were observed between 250 and 500  $\mu\text{g/mL}$  (Fig. 3). With regard to the restoration of ALP activity, no significant differences were observed up to a concentration of 250  $\mu\text{g/mL}$ . At 500  $\mu\text{g/mL}$ , the restorative ability significantly decreased after a 2-week culture without GM. At 750 and 1000  $\mu\text{g/mL}$ , the restorative ability remained low, although no significant difference was observed between the two concentrations. At 2000  $\mu\text{g/mL}$ , there was a significant difference in restorative ability, although the extent was small (Fig. 4).

**Mineralization assay:** Compared to 0  $\mu\text{g/mL}$  GM, the mineralization potential was significantly reduced in all samples at  $\geq 100$   $\mu\text{g/mL}$ ; compared to 100  $\mu\text{g/mL}$  GM, the mineralization potential was significantly reduced in all samples at  $\geq 500$   $\mu\text{g/mL}$ ; compared to 250  $\mu\text{g/mL}$ , all were significantly decreased at  $\geq 750$   $\mu\text{g/mL}$ ; compared to 500  $\mu\text{g/mL}$ , all were significantly decreased at  $\geq 1000$   $\mu\text{g/mL}$  (Fig. 5).

**DISCUSSION:** CLAP therapy in clinical practice generally involves continuous administration of 1200  $\mu\text{g/mL}$  GM, and the average duration of CLAP therapy is approximately two weeks [1]. Based on the results of this study, it is possible that the current clinical dose may have adverse effects on cells, and that these effects may persist after the end of exposure. However, the reported bone union rate after CLAP therapy is only 95% [3]; therefore, the GM solution may be diluted for exudates or hematomas. Further studies are required to determine the optimal antibiotic concentration for CLAP therapy.

**SIGNIFICANCE:** The current study demonstrates that high concentrations of GM negatively affect cell function. These results provide an important basis for selecting optimal concentrations of GM for CLAP therapy.

**REFERENCES:** [1] Maruo A, et al. J Orthop Surg. 2021. [2] Oe K, et al. J Orthop Case Rep. 2021. [3] Maruo A, et al. J Orthop Surg. 2022. [4] Sawauchi K, et al. Ultrasound Med Biol. 2022.

