Bone & Joint Research

Supplementary Material

10.1302/2046-3758.133.BJR-2023-0198.R1

Table i. Clinical data of the study patients.

Patient	Sex	Age, yrs	Clinical diagnosis
1	M	69	Infected nonunion/tibia
2	M	63	Infected nonunion/tibia
3	F	71	Infected nonunion/tibia
4	F	64	Pyogenic arthritis/ankle Acute osteomyelitis/tibia
5	F	67	Acute osteomyelitis/tibia
6	M	61	Acute osteomyelitis/calcaneus

F, female; M, male.

Table ii. Gene-specific primer sequences for real-time reverse transcription-polymerase chain reaction.

Gene	Primer sequence (5′ to 3′) (forward/reverse)
RUNX2	ATGCTTCATTCGCCTCACAAAC
	CCAAAAGAAGTTTTGCTGACATGG
OSX	CGGGACTCAACAACTCT
	CCATAGGGGTGTGTCAT
ATF4	CTGACCACGTTGGATGACAC
	GGGCTCATACAGATGCCTCT
COL1A1	AGGAATTCGGCTTCGACGTT
	GGTTCAGTTTGGGTTGCTTG
OC	CATGAGAGCCCTCACA
	AGAGCGACACCCTAGAC
BMP2	ACCAGACTATTGGACACCAG
	AATCCTCACATGTCTCTTGG
ALP	CTCGTTGACACCTGGAAGAGCTTCAAACCG
	GGTCCGTCACGTTGTTCCTGTTCAGC
GAPDH	CGTCTTCACCACCATGGAGA
	CGGCCATCACGCCACAGTTT

ALP, alkaline phosphatase; ATF4, activating transcription factor 4; BMP2, bone morphogenetic protein 2; COL1A1, collagen type I; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OC, osteocalcin; OSX, osterix; RUNX2, runt-related transcription factor 2.

Methods

Live cells rate (cell viability)

Live cells rate (cell viability) was measured with the water-soluble tetrazolium salt assay using the cell counting kit-8 (CCK-8) (Dojindo, Japan). On days 4, 7, and 14 after the start of exposure, 10 µl of water-soluble tetrazolium salt was added to each well containing cells, incubated at 37°C for four hours, and the soluble formazan was quantified in viable cells by measuring the absorbance of the reduced formazan at 450 nm. The live cells rate at each timepoint was calculated by setting that at the start of exposure as 100%.

Apoptosis rate assay

Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining using the APO-DIRECT kit (Phoenix Flow Systems, USA) 48 hours after gentamicin (GM) exposure, according to the manufacturer's protocol. The rate of apoptosis was calculated as the ratio of green-stained nuclear fragments to 4′,6-diamidino-2-phenylindole (DAPI)-stained cells for each of the four fields of view.

ALP activity assay

Sonicated cells that were stored at -20°C were used to assess ALP activity. ALP activity was evaluated as the release of p-nitrophenol from p-nitrophenyl phosphate, pH 9.8, which was monitored by measuring absorbance at 405 nm using the SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Corp, USA). The protein concentration was standardized using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, USA).

Real-time reverse transcription-polymerase chain reaction

The total RNA of the cultured cells was extracted using the RNeasy Mini Kit (Qiagen, USA) on days 0, 7, and 14. The total RNA was reverse transcribed into single-strand complementary DNA using a high-capacity complementary DNA reverse transcription kit (Applied Biosystems, USA). Measurements were performed in duplicate using an Applied Biosystems 7,500 real-time polymerase chain reaction (PCR) system, and the primers were purchased from Thermo Fisher Scientific (USA).

Mineralization assay

Mineralization assays were performed to evaluate the calcification of the extracellular matrix during osteogenic differentiation. The cells were cultured in a GM-containing medium for one week followed by that in a GM-free medium for three weeks. The plate was stained with 1% alizarin red S (Hartman Leddon, USA), wherein the mineralized extracellular matrix appeared red.

In addition to the above, the cells were stained with alizarin red S (Iwai Chemicals, Japan) for 30 minutes. The calcium content was detected using a colorimetric method. Formic acid (2%; Iwai Chemicals) was added to each well, and the plate was incubated at room temperature for ten minutes. The alizarin red S concentration was evaluated with the absorbance at 405 nm and expressed as relative intensity levels compared with that of the 0 μg/ml GM concentration group on day 28.

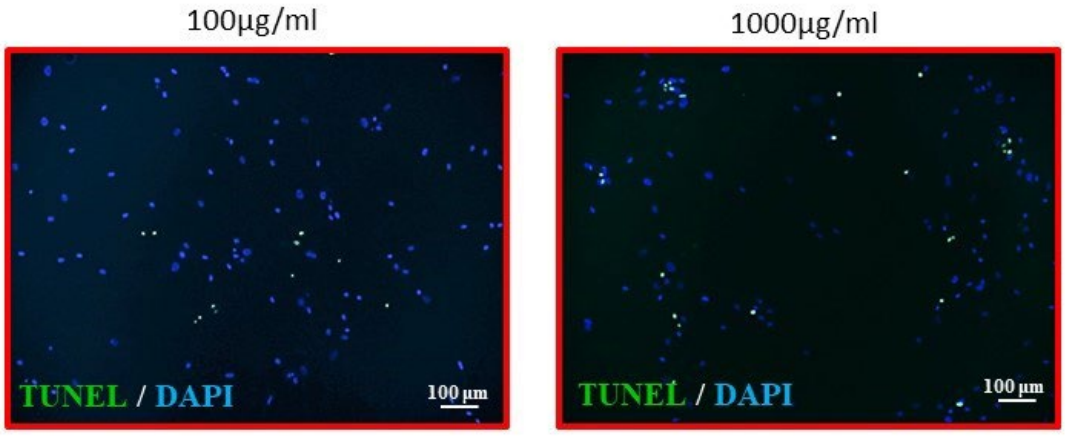

Fig a. Fluorescent-stained microscopic images. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and 4′,6-diamidino-2-phenylindole (DAPI) staining are shown as green and blue, respectively.

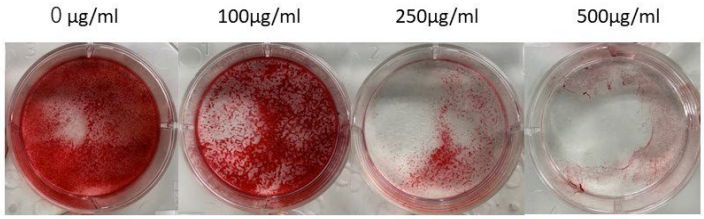

Fig b. Representative images of the results of Alizarin Red S staining at each gentamicin (GM) concentration.