Lovastatin increases the proliferation and osteoblastic differentiation of human gingiva-derived stem cells in three-dimensional cultures

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Abstract. Lovastatin is a cholesterol-lowering agent that also has effects of cell proliferation and apoptosis. The present study was performed to evaluate the effects of lovastatin on the proliferation and osteogenic differentiation of three-dimensional cell spheroids formed from human gingiva-derived stem cells (GDSCs) using concave microwells. GDSCs were plated on polydimethylsiloxane-based concave micromolds and grown in the presence of lovastatin at concentrations of 0, 2 and 6 μM. The morphology of the cells was viewed under an inverted microscope, and cell viability was determined with Cell Counting kit-8 on days 2, 7 and 14. Alkaline phosphatase activity assays were performed to evaluate the osteogenic differentiation on days 2 and 8. Alizarin red-S staining was also used to assess the mineralization of the stem cell spheroids at day 14. The results confirmed that GDSCs formed spheroids in concave microwells. No significant changes were noted with longer incubation time, and no significant differences in cell viability were noted between the three lovastatin groups at each time point. Higher osteogenic differentiation was observed in the 2 μM group when compared with the control. Mineralized extracellular deposits were visible after Alizarin red-S staining, and higher mineralization was noted in the 2 and 6 μM lovastatin groups when compared with the 0 μM control. The relative mineralization values of the 0, 2 and 6 μM groups on day 14 were 39.0±9.6, 69.3±6.0 and 60.9±7.5, respectively. This study demonstrated that the application of lovastatin enhanced the osteogenic differentiation of cell spheroids formed from GDSCs. This suggests that combinations of lovastatin and stem cell spheroids may have the potential for use in tissue engineering.

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Introduction

The majority of cell culture experiments are conducted on two-dimensional surfaces, including micro-well plates, tissue culture flasks and Petri dishes, due to the ease and convenience of two-dimensional cultures (1). However, two-dimensional cultures may have limitations for the evaluation of cell and tissue physiology, including the communication between a cell and its matrix and between adjacent cells (2). To overcome these limitations, three-dimensional culture techniques have been applied (3,4). Three-dimensional cultures have advantages including the ability to represent *in vivo* morphologies and the potential for use in drug discovery with primary and stem cells (5). In a previous study, three-dimensional culture platforms were made using engineered microenvironments, such as highly porous biomimetic scaffolds, which exhibited higher cell differentiation efficiency compared with their two-dimensional counterparts (4). Furthermore, three-dimensional cultures have been shown to support the long-term expansion of nephrogenic progenitor cells (6). Gingiva-derived stem cells (GDSCs) display multipotency with high proliferation characteristics (7,8)

Lovastatin is a cholesterol-lowering agent (9); it is involved in regulation of the mevalonate pathway and also affects Akt pathways that are involved in cell proliferation and apoptosis, leading to antiproliferative effect (10). However, the effects of lovastatin on mesenchymal stem cells with three-dimensional cultures have not been well elucidated. Therefore, the purpose of the present study was to evaluate the effects of lovastatin on the proliferation and osteogenic differentiation of human gingiva-derived stem cells (GDSCs) using concave microwells. To the best of the authors' knowledge, this investigation is the first to elucidate the effects of lovastatin on three-dimensional spheroid cultures using mesenchymal stem cells derived from gingiva.

Materials and methods

Isolation and culture of human GDSCs. Gingival tissues were collected from 75-year-old female undergoing periodontal surgery on August 2013 at Seoul St Mary's Hospital, College of Medicine, The Catholic University of Korea. The design of the study was reviewed and approved by the Institutional Review Board of the Catholic University of Korea, College of

Medicine (no. KC11SISI0348). Informed consent was obtained from all participants according to the Act on Legal Codes for Biomedical Ethics and Safety and the Declaration of Helsinki. Human GDSCs were isolated and cultivated following the protocol published in the present authors' previous study (7). The gingival tissues were collected and maintained in sterile phosphate-buffered saline (PBS; Welgene, Inc.) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 4˚C. The tissues were de-epithelialized, separated into 1-2-mm^2 fragments, 0.2 μm filtered, and digested in modified in α-minimum essential medium (α-MEM; Gibco; Thermo Fisher Scientific, Inc.) containing dispase (1 mg/ml; Sigma-Aldrich; Merck KGaA) and collagenase type IV (2 mg/ml; Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min. The cell suspension was filtered with a 70-μm cell strainer (Falcon; BD Biosciences) and the cells were then incubated at 37˚C in a humidified incubator with 5% CO_2. After 24 h, the non-adherent cells were washed with PBS.

Formation of spheres and evaluation of cellular morphology. Fig. 1 demonstrates the overview of the present study design. Cells were plated onto silicon elastomer-based concave microwells (StemFIT 3D; MicroFIT) of 600 μm diameter at a density of 4x10^5 cells/well and cultured in osteogenic media (StemPro® Osteogenesis Differentiation Kit; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C. The medium was refreshed at 3-day intervals. To examine the effect of lovastatin, the cells were cultured in the presence of lovastatin (Abcam) at final concentrations of 0 (untreated control), 2 and 6 μM using dimethyl sulfoxide (DMSO) as the vehicle at plating. The concentrations of lovastatin used in the present study were based on those used in previously published studies (11-14). Equal amounts of DMSO were added to each culture sample to offset the influence of this dissolving vehicle (15). The cells expressed CD44 surface marker and the cell spheroids were positive for SSEA-4 (7,16). The morphology of the microspheres was viewed under an inverted microscope (CKX41; Olympus Corporation) on days 2, 7 and 14 following plating. The diameter of the cell spheroids was measured at each time point.

Determination of cytotoxicity. The cytotoxicity of lovastatin was evaluated on days 2, 7 and 14 with a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. The absorbance at 450 nm was measured spectrophotometrically using a microplate reader (BioTek Instruments, Inc.).

Alkaline phosphatase activity assays. Stem cell spheroids grown with osteogenic media (StemPro® Osteogenesis Differentiation kit; Gibco; Thermo Fisher Scientific, Inc.) were obtained on day 2 and 8. Alkaline phosphatase activity assays were tested using a commercially available kit (K412-500, BioVision, Inc., Milpitas, CA, USA) following the manufacturer's protocol. The cells were resuspended with an assay buffer, sonicated and then centrifuged at 15,000 x g for 10 min at 4˚C to remove insoluble material. The supernatant was mixed with p-nitrophenylphosphate substrate and incubated at 25˚C for 60 min. The optical density was determined spectrophotometrically at 405 nm.
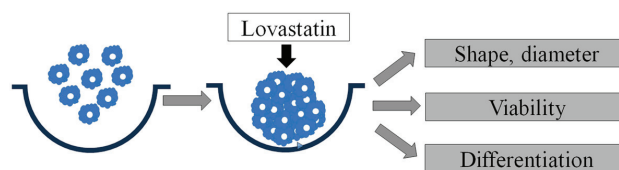

Figure 1. Schematic illustration of the three-dimensional culture and analysis of gingival-derived stem cells.

Alizarin red-S staining. To investigate mineralized nodule formation, cells were grown with osteogenic media (StemPro® Osteogenesis Differentiation kit; Gibco; Thermo Fisher Scientific, Inc.) for 14 days. The cell spheroids were fixed with 4% paraformaldehyde at room temperature and stained with Alizarin red-S (ScienCell Research Laboratories, Inc.) at room temperature for 30 min. Inverted microscopy (CKX41) was used for evaluation of the stained cells (magnification, x100). The relative value of mineralization was determined by measuring the relative intensity of staining using image processing and analysis software (ImageJ version 1.8.0; National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard deviation with 95% confidence of intervals (95% CI). Experiments were performed at least three times. A test of normality was performed with a Shapiro-Wilk test. Two-way analysis of variance (ANOVA) was performed for evaluation of the effects of concentration and time and one-way ANOVA was used to determine the differences among groups, followed by Tukey's post hoc test. The analysis was conducted with SPSS 12 for Windows (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant result.

Results

Evaluation of cell morphology and cellular viability. GDSCs formed spheroids in the concave microwells. The morphology of the spheroids at day 2 is shown in Fig. 2A-C. The morphologies of the spheroids at days 7 and 14 were similar to those at day 2 (Fig. 2D-I). No obvious changes in morphology were observed as the incubation time increased. The diameters of the spheroids were smallest in the 6 μM group at day 2 (P<0.001; Fig. 3). The average diameters of the stem cell spheroids at day 2 were 309.9±32.7 (95% CI: 280.5-339.2), 331.2±24.0 (95% CI: 301.9-360.6) and 231.4±24.4 (95% CI: 202.0-260.7) μm for 0, 2 and 6 μM lovastatin, respectively (P=0.001). The average diameters at day 7 were 322.2±27.1 (95% CI: 292.9-351.5), 376.4±27.9 (95% CI: 347.1-405.8) and 235.9±29.6 (95% CI: 206.6-265.2) μm for 0, 2 and 6 μM lovastatin, respectively (P<0.001). The average diameters at day 14 were 275.8±33.6 (95% CI: 246.4-305.1), 346.4±11.8 (95% CI: 317.1-375.8) and 317.7±38.2 (95% CI: 288.3-347.0) μm for 0, 2 and 6 μM, respectively (P=0.027; Table I). In general, the diameters of the spheroids were maintained throughout the incubation period.

Cell cytotoxicity. Cell cytotoxicity was measured for the spheroids after culturing for 2, 7 and 14 days (P=0.035; Fig. 4). The CCK-8 assay results for the 0, 2 and 6 μM groups on
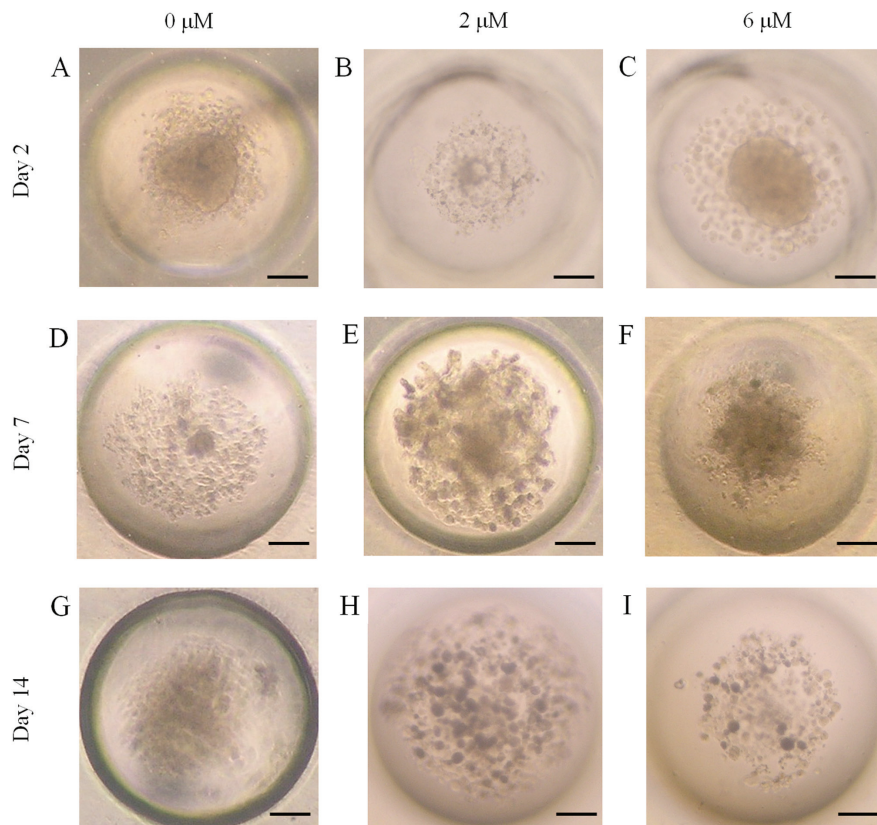
Figure 2. Evaluation of the morphology of the three-dimensional stem cells on days 2, 7 and 14. (A) 0 μM, (B) 2 μM and (C) 6 μM lovastatin groups on day 2; (D) 0 μM, (E) 2 μM and (F) 6 μM lovastatin groups on day 7; (G) 0 μM, (H) 2 μM and (I) 6 μM lovastatin groups on day 14. Scale bar, 100 μm.
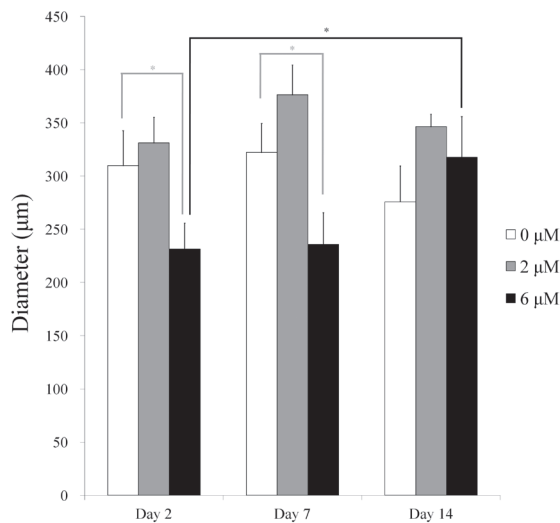

Figure 3. Evaluation of the diameters of the cell spheroids with 0, 2 and 6 μM lovastatin on days 2, 7 and 14. *P<0.05, with comparisons indicated by lines.

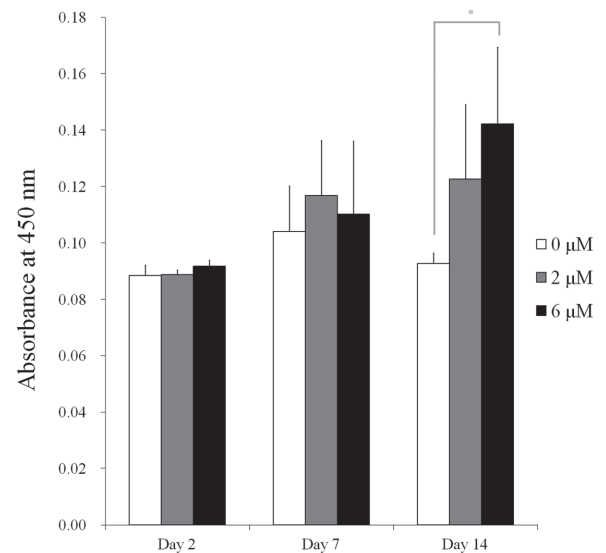

Figure 4. Cellular viability of the cell spheroids with 0, 2 and 6 μM lovastatin on days 2, 7 and 14. *P<0.05, with comparisons indicated by lines.

day 2 were 0.088±0.004 (95% CI: 0.073, 0.104), 0.089±0.001 (95% CI: 0.073, 0.105) and 0.092±0.002 (95% CI: 0.076, 0.108), respectively (P=0.108). The CCK-8 assay results at day 7 were 0.104±0.016 (95% CI: 0.088, 0.120), 0.117±0.019 (95% CI: 0.101, 0.133) and 0.110±0.026 (95% CI: 0.094, 0.126) for the 0, 2 and 6 μM groups, respectively (P=0.634). No statistically significant differences were detected between the groups at days 2 and 7 (P>0.05). The CCK-8 assay values at

day 14 were 0.093±0.004 (95% CI: 0.077, 0.108), 0.123±0.026 (95% CI: 0.107, 0.138) and 0.142±0.027 (95% CI: 0.126, 0.158) for the 0, 2 and 6 μM groups, respectively (P=0.012; Table II).

Alkaline phosphatase activity assay. The results of the alkaline phosphatase activity assay on days 2 and 8 are shown in Fig. 5. The absorbance values at 405 nm on day 2 for the

Table I. Cell spheroid diameters (μm) for various lovastatin concentrations at different time points.

| Time point	Lovastatin		
	0 μM	2 μM	6 μM
Day 2	309.9±32.7 (280.5-339.2)	331.2±24.0 (301.9-360.6)	231.4±24.4 (202.0-260.7)
Day 7	322.2±27.1 (292.9-351.5)	376.4±27.9 (347.1-405.8)	235.9±29.6 (206.6-265.2)
Day 14	275.8±33.6 (246.4-305.1)	346.4±11.8 (317.1-375.8)	317.7±38.2 (288.3-347.0)

Values are presented as the mean ± SD (95% confidence interval).

Table II. Cytotoxicity of the cell spheroids for various lovastatin concentrations at different time points.

Time point	Lovastatin		
	0 μM	2 μM	6 μM
Day 2	0.088±0.004 (0.073, 0.104)	0.089±0.001 (0.073, 0.105)	0.092±0.002 (0.076, 0.108)
Day 7	0.104±0.016 (0.088, 0.120)	0.117±0.019 (0.101, 0.133)	0.110±0.026 (0.094, 0.126)
Day 14	0.093±0.004 (0.077, 0.108)	0.123±0.026 (0.107, 0.138)	0.142±0.027 (0.126, 0.158)

Values are presented as the mean ± SD (95% confidence interval).
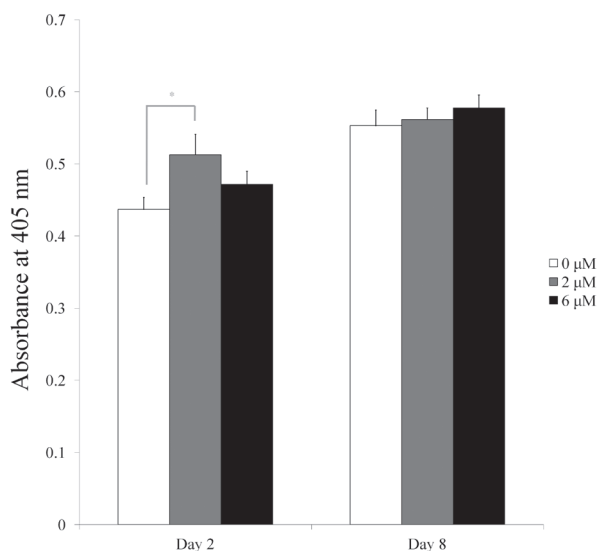
Figure 5. Alkaline phosphatase activity of the cell spheroids on days 2 and 8. *P<0.05, with comparisons indicated by lines.

0, 2 and 6 μM groups were 0.437±0.017, 0.513±0.028 and 0.472±0.018, respectively (P<0.05). The value for the 2 μM group was significantly higher compared with that of the 0 μM group (P<0.05). The absorbance values at 405 nm on day 8 for the 0, 2 and 6 μM groups were 0.553±0.022, 0.562±0.016 and 0.578±0.018, respectively (P>0.05).

Mineralization assay. Mineralized extracellular deposits were observed after Alizarin red-S staining on day 14 (Fig. 6). Higher mineralization was observed in the 2 and 6 μM groups

when compared with the 0 μM control (Fig. 7; P<0.05). The relative values of the 0, 2 and 6 μM groups on day 14 were 39.0±9.6 (95% CI: 23.6, 54.3), 69.3±6.0 (95% CI: 59.8, 78.8) and 60.9±7.5 (95% CI: 49.0, 72.8), respectively (P=0.001).

Discussion

The present study clearly demonstrates that lovastatin at the tested concentrations did not adversely affect the viability of the stem cell spheroids, and increased their osteogenic differentiation.

Statins are drugs that are widely used for lowering serum cholesterol, but have also been shown to enhance new bone formation *in vitro* and in rodents in previous studies (15,17-19). The locally delivery of lovastatin using biodegradable polymer nanobeads of poly(lactic-co-glycolide acid) has been found to improve fracture healing in rats (20). In another study, lovastatin-loaded biodegradable polyurethanes exhibited sustained release of biologically active lovastatin, and the released lovastatin significantly enhanced the osteogenic differentiation of osteoblastic cells *in vitro* (21). Similarly, the present study using cell spheroids without scaffold confirmed the increased osteogenic differentiation of stem cells when cultured with 2-6 μM lovastatin, suggesting a potential application in therapeutic agents for bone formation.

In a previous study, statins impaired the survival of primary human mesenchymal progenitor cells via mevalonate depletion, nuclear factor κB signaling and B-cell lymphoma 2/adenovirus E1B 19 kDa protein-interacting protein 3 when 1 and 10 μM simvastatin or atorvastatin was used (22). In another study, exposure to simvastatin (0-20 μM) induced a reduction in sphere-forming capacity and cell viability,

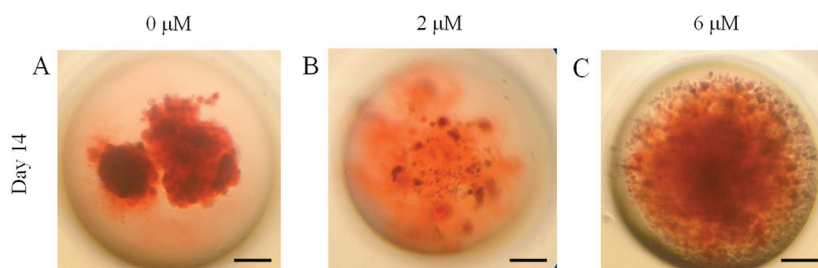Figure 6. Representative images of Alizarin red-S staining on day 14. (A) 0 μM, (B) 2 μM and (C) 6 μM lovastatin groups on day 14. Scale bar, 100 μm.
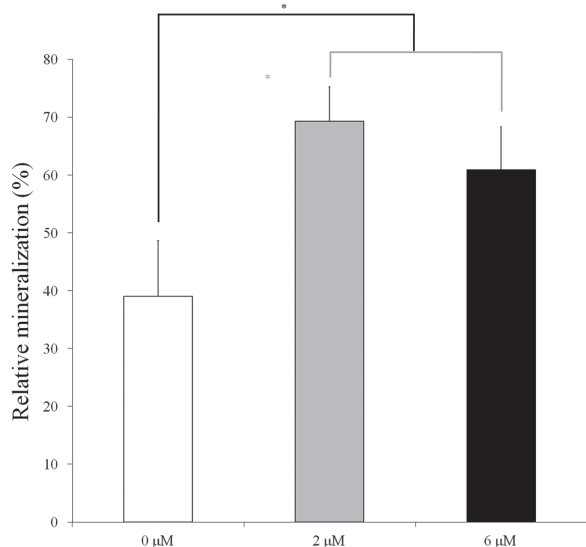

Figure 7. Quantitative results of the mineralization assay. *P<0.05, with comparisons indicated by lines.

accompanied by a concentration- and time-dependent increase in caspase-3/7 activity (23). Conversely, lovastatin (0.01-1 μM) was found to prevent mesenchymal stem cells from undergoing hypoxia/serum deprivation-induced apoptosis through inhibition of the mitochondrial apoptotic pathway, leading to attenuation of caspase-3 activation (24). In an *in vitro* study, simvastatin inhibited mesenchymal stem cell apoptosis and increased vascular endothelial growth factor, and combined treatment with simvastatin and mesenchymal stem cells induced a significant improvement in blood reperfusion and a notable increase in capillary density (25).

Mesenchymal stem cells have been applied in tissue engineering, for tissues including bone, cartilage, fat and other connective tissue (26). Mesenchymal stem cells have been characterized from a variety of dental-related tissues, including periodontal ligaments, papilla, follicle, dental pulp of exfoliated deciduous and adult teeth, and the maxillary sinus membrane, which represent rich sources of mesenchymal stem cells (27,28). These stem cells have the capacity for self-renewal and multi-lineage differentiation, including osteogenic, chondrogenic and adipogenic differentiation (29). In addition, dental stem cells display several advantages, including a high proliferation rate, high viability and easy induction to distinct cell lineages (27). Moreover, human GDSCs can be harvested during routine practice under local anesthesia and

may be considered an excellent source for tissue-engineering purposes (7). Further studies are warranted to evaluate the effects of combination therapy using animal models.

The present study demonstrated that cell spheroids formed from stem cells combined with the application of lovastatin at the tested concentrations had enhanced osteogenic differentiation capability. Therefore, combinations of lovastatin and stem cell spheroids may potentially be useful for tissue engineering purposes.

Acknowledgements

Availability of data and materials

All data generated or analyzed during the present study are included in the published article.

Authors' contributions

BK, JT, YK and JP designed the study, performed the experiments, were responsible for data collection and analysis, and participated in drafting the manuscript. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures involving human participants were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards, and were approved by the Institutional Review Board of the Catholic University of Korea, College of Medicine (no. KC11SISI0348). Informed consent was obtained from the participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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