Arbutin promotes MC3T3-E1 mouse osteoblast precursor cell proliferation and differentiation via the Wnt/β-catenin signaling pathway

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Abstract. Arbutin is a natural compound extracted from various plants, including bearberry leaves, that exerts multiple effects including skin whitening, anti-inflammatory and oxidative stress-protective properties. However, the effects of arbutin on osteoblasts remain unknown. The aim of the present study was to investigate the function and the mechanisms of arbutin on the proliferation and differentiation of MC3T3-E1 mouse osteoblast precursor cells in vitro. The proliferation of MC3T3-E1 cells treated with arbutin was assessed using a Cell Counting Kit-8 assay and a 5-ethynyl-2'-deoxyuridine labeling assay. Additionally, cell cycle and apoptosis were examined using flow cytometry analysis. The effects of arbutin on osteoblast differentiation were investigated using alkaline phosphatase (ALP) staining and by examining the mRNA expression of osteoblast differentiation. The effects of arbutin on osteoblasts remain unknown. The aim of the present study was to investigate the function and the mechanisms of arbutin on the proliferation and differentiation of MC3T3-E1 mouse osteoblast precursor cells in vitro. The proliferation of MC3T3-E1 cells treated with arbutin was assessed using a Cell Counting Kit-8 assay and a 5-ethynyl-2'-deoxyuridine labeling assay. Additionally, cell cycle and apoptosis were examined using flow cytometry analysis. The effects of arbutin on osteoblast differentiation were investigated using alkaline phosphatase (ALP) staining and by examining the mRNA expression levels of collagen type I α1 chain (COL1A1), bone γ-carboxyglutamate protein (BGLAP) and Sp7 transcription factor (SP7). To further investigate the molecular mechanism underlying arbutin function in promoting osteogenesis, the mRNA and protein expression levels of runt-related transcription factor 2 (RUNX2) and β-catenin were analyzed by reverse transcription-quantitative polymerase chain reaction and western blotting. Arbutin significantly promoted MC3T3-E1 cell proliferation and increased the ratio of cells in S-phase. Treatment with arbutin increased ALP activity and the mRNA expression levels of COL1A1, BGLAP and SP7 in MC3T3-E1 cells. Furthermore, the protein and the mRNA expression levels of RUNX2 and β-catenin increased significantly following treatment with arbutin. Collectively, the present findings suggested that arbutin was able to promote proliferation and differentiation of MC3T3-E1 cells via the Wnt/β-catenin signaling pathway.

Introduction

Osteoporosis is a health and socioeconomic problem characterized by low bone mass and deteriorated bone microarchitecture, which increases the risk of fragility fractures (1,2). In the European Union (EU), the prevalence rate of osteoporosis in individuals aged ≥50 years was estimated at 6.6 and 22.1% for men and women in 2010, respectively. Economically, the total cost of osteoporosis to the EU was ~€37.4 billion in 2010 (3). Owing to the increase in life expectancy and a growing aging population, an increasing number of people may suffer from osteoporotic fractures in the future (4). Osteoporosis is caused by an imbalance between bone formation, mediated by osteoblasts, and resorption, mediated by osteoclasts (5). Available osteoporosis treatments include anti-resorption drugs (such as bisphosphonates and denosumab), calcitonin and estrogen. However, these compounds exhibit certain limitations; in particular, they decrease the osteogenic turnover rate, and by decreasing the bone-remodeling process, they cause a decrease in bone formation (6). Estrogen therapy is not ideal for long-term osteoporosis therapy, since high levels of estrogen may induce uterine bleeding, breast cancer and cardiovascular diseases (7). In addition, anti-resorption drugs are not able to restore lost bone structure. However, anabolic agents may stimulate bone formation and increase bone mass (8). Therefore, it is important to identify novel safe and effective drugs able to promote bone formation.

Arbutin (4-hydroxyphenyl-β-D-glucopyranoside; Fig. 1) is a naturally occurring hydroquinone derivative (molecular mass 272 Da) present in various types of plants. High levels of arbutin were identified in plants including, marjoram (Origanum majorana, Lamiaceae) and pears (Pyrus communis, Rosaceae) and, in particular, the Ericaceae family such as bearberry leaves (Arctostaphylos uva-ursi) (9). Arbutin exhibits various biological activities. For example, arbutin may be used as a skin whitening agent; by inhibiting the tyrosinase activity in melanosomes, arbutin was identified to promote depigmentation (10,11). A previous study demonstrated that arbutin may serve a protective role from X-irradiation-induced apoptosis by decreasing the intracellular levels of hydroxyl radicals (12). Additionally, arbutin may inhibit osteoclast differentiation by decreasing the intracellular levels of superoxide and by downregulating nuclear factor of activated T cells 1 (13). However, the effects of arbutin on osteoblast function remain unknown.
unknown. Therefore, the present study aimed to investigate the effects and the mechanisms of arbutin on MC3T3-E1 mouse osteoblast precursor cell proliferation and differentiation.

The Wnt signaling pathway may affect osteoblast and osteoclast, directly and indirectly, increasing bone formation and decreasing bone resorption (14). Canonical Wnt signaling may regulate proliferation, differentiation and function of osteoblast at multiple levels (15). In animal models, decreasing the activity of inhibitors of the Wnt/β-catenin signaling pathway by using antibodies against secreted frizzled protein-related protein 1, sclerostin and dickkopf WNT signaling pathway inhibitor 1 (DKK1), and small-molecule inhibitors of glycosgen synthase kinase 3 β (GSK-3β) may increase bone mass and decrease the risk of fractures (16). Therefore, the Wnt signaling pathway represents a potential therapeutic target for the development of novel drugs to treat osteoporosis (17). In the present study, the effects of arbutin on MC3T3-E1 cell proliferation and differentiation were investigated. In addition, the molecular mechanism underlying arbutin function in inducing MC3T3-E1 cell differentiation was examined.

Materials and methods

Chemicals. Arbutin (purity, ≥98%) was purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China), dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and stored at a concentration of 0.5 M. Recombinant human DKK1 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA; cat. no. 120-30).

Cell culture. MC3T3-E1 mouse calvarial pre-osteoblasts were purchased from The Cell Resource Center of The Shanghai Institutes for Biological Sciences of The Chinese Academy of Sciences (Shanghai, China), and were cultured in α-Minimum Essential Medium (α-MEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 100 µg/ml streptomycin and 100 U/ml penicillin (HyClone; GE Healthcare Life Sciences). Cells were maintained in a cell culture incubator with 5% CO₂ at 37°C. The medium was replaced every other day. Cells at 80% confluence were reseeded into tissue culture flasks following treatment with osteogenic supplement and 2% (v/v) dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 2 h at 37°C. For osteoblastic differentiation experiments, cells were treated with osteogenic supplement containing 50 µg/ml L-ascorbic acid (Sigma-Aldrich; Merck KGaA) and 10 mM β-glycerophosphate disodium salt hydrate (Sigma-Aldrich; Merck KGaA) for 9 days at 37°C. For mechanistic studies, MC3T3-E1 cells were pretreated with DKK1 (0.5 µg/ml) for 6 h at 37°C, and were then treated with arbutin (100 µM) for 3 days at 37°C.

Cell proliferation. A Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess the effects of arbutin on cell proliferation. Cells were seeded in 96-well plates at a density of 5x10⁴ cells/well for 24 h at 37°C. Subsequently, cells were treated with arbutin at various concentrations (0, 10, 50 and 100 µM). After 24, 48 or 72 h, cells were treated with a 9.1% CCK-8 solution containing 10 µl CCK-8 and 100 µl α-MEM for 1-2 h at 37°C. The optical density value of each well was measured using a microplate reader (ELX808; BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 nm. Relative cellular viability was calculated as the ratio between the mean absorbance of the sample and the control.

5-ethynyl-2′-deoxyuridine (EdU) labeling assay. The effect of arbutin on cell proliferation was measured using an EdU Apollo®567 in vitro Imaging kit (Guangzhou Ribio Bio Co., Ltd., Guangzhou, China). Cells were inoculated in a 96-well plate at a density of 1x10⁵ cells/well and incubated in α-MEM containing 10% FBS with 0, 10, 50 or 100 µM arbutin. Following a 72-h incubation, EdU was added to each well at a concentration of 50 µM prior to an additional incubation for 2 h at 37°C. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature (RT). Following washing with glycine (2 mg/ml) and PBS, cells were permeabilized with Triton X-100 (0.5%) for 10 min at RT. Cells were incubated with 1X Apollo staining reaction liquid at RT for 30 min in the dark. Cell nuclei were counter-stained with 1X Hoechst 33342 for 30 min at RT. EdU-positive cells were visualized by fluorescence microscopy (magnification, x200; Eclipse Ti; Nikon Corporation, Tokyo, Japan) in five randomly selected fields.

Cell cycle and apoptosis analysis. MC3T3-E1 cells were seeded in six-well plates at a density of 2x10⁵ cells/well. Following a 24-h incubation, cells were treated with arbutin at concentrations of 0, 10, 50 and 100 µM. Cells were harvested after 3 days at RT and fixed with 70% ethanol for 12 h at 4°C. Cells were washed three times with PBS and stained with propidium iodide (PI) staining solution (Beyotime Institute of Biotechnology, Haimen, China) for 30 min at 37°C in the dark. DNA content was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with CellQuest Pro software (Version 5.2; BD Biosciences) and ModFit LT software (Version 3.0; Verity Software House, Inc., Topsham, ME, USA). For apoptosis analysis, cells treated with arbutin were harvested and stained with fluorescein isothiocyanate-labeled Annexin-V and PI (Dojindo Molecular Technologies, Inc.) in the dark for 15 min at RT. The cell apoptotic rate was assessed using a FACSCalibur flow cytometer (BD Biosciences) with CellQuest Pro software (Version 5.2; BD Biosciences).

Alkaline phosphatase (ALP) staining assay. Osteoblasts were seeded in 6-well plates at a density of 5x10⁴ cells/well incubated in α-MEM containing osteogenic supplement and treated with 0 (control), 10, 50 or 100 µM arbutin. After 9 days at 37°C, cells were washed three times with PBS and fixed in 4% paraformaldehyde at RT for 10 min. Cells were rinsed three times with distilled water and subsequently stained using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride ALP color development kit (Beyotime Institute of Biotechnology) for 2 h at RT. Stained cells were imaged using a light microscope (magnification, x40; Eclipse Ti; Nikon Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were seeded in 6-well plates at a density of 2x10⁵ cells/well. Following treatment with arbutin at various
concentrations for 3 days at 37°C, total RNA was extracted from MC3T3-E1 cells using RNAsiso Plus reagent (Takara Biotechnology Co., Ltd., Dalian, China). In total, 1 µg RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.), according to the manufacturer’s instructions. The reaction conditions were as follows: 42°C for 2 min, 37°C for 15 min and 85°C for 5 sec. qPCR was performed using equal amounts of cDNA from each sample in a total volume of 20 µl with an ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using SYBR premix Ex Taq II (Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used: Initial denaturation at 95°C for 30 sec, followed by 30 cycles of 95°C for 5 sec and 60°C for 34 sec. Specificity of the amplification was assessed by melting curve analysis, and β-actin served as an internal control. Relative gene expression levels were analyzed using the 2^(-ΔΔCt) method (18). The sequences of the primers used were the following: Runt-related transcription factor 2 (RunX2), forward 5'-CCAACCGATCATTGAAAGCT-3', reverse 5'-GCTCACGTGCATCTTGG-3'; collagen type I α chain (COL1A1), forward 5'-GGCCCTCCAGAACATCTACCT3'-3', reverse 5'-GCAGGAGCCTTTGAGTTGG-3'; bone γ-carboxyglutamate protein (BGLAP), forward 5'-CCGATTGGAGCCTAGT-3', reverse 5'-AGGAGTTGCTTACAAGGATAC3'-3'; β-actin, forward 5'-ATGGAGCGGACAGAACAG3'-3', reverse 5'-CTGGCCACTCGAGAAGGA-3'; β-actin, forward 5'-GGCTGTATTGCCTCCTAC3'-3', reverse 5'-CCAGTTGGTAAAACATGCGGACTT-3'.

**Western blot analysis.** MC3T3-E1 cells were seeded in 6-well plates at a density of 2x10⁵ cells/well. Cells were treated with arbutin at various concentrations for 3 days and rinsed three times with ice-cold PBS. Total cellular protein was extracted from MC3T3-E1 cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing 1 mM phenylmethylsulfonyl fluoride. Proteins were isolated following a centrifugation at 13,800 x g for 15 min at 4°C. Protein concentration was quantified using a bicinchoninic acid reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.), according to the manufacturer’s instructions. The reaction conditions were as follows: 42°C for 2 min, 37°C for 15 min and 85°C for 5 sec. qPCR was performed using equal amounts of cDNA from each sample in a total volume of 20 µl with an ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using SYBR premix Ex Taq II (Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used: Initial denaturation at 95°C for 30 sec, followed by 30 cycles of 95°C for 5 sec and 60°C for 34 sec. Specificity of the amplification was assessed by melting curve analysis, and β-actin served as an internal control. Relative gene expression levels were analyzed using the 2^(-ΔΔCt) method (18). The sequences of the primers used were the following: Runt-related transcription factor 2 (RunX2), forward 5'-CCAACCGATCATTGAAAGCT-3', reverse 5'-GCTCACGTGCATCTTGG-3'; collagen type I α chain (COL1A1), forward 5'-GGCCCTCCAGAACATCTACCT3'-3', reverse 5'-GCAGGAGCCTTTGAGTTGG-3'; bone γ-carboxyglutamate protein (BGLAP), forward 5'-CCGATTGGAGCCTAGT-3', reverse 5'-AGGAGTTGCTTACAAGGATAC3'-3'; β-actin, forward 5'-ATGGAGCGGACAGAACAG3'-3', reverse 5'-CTGGCCACTCGAGAAGGA-3'; β-actin, forward 5'-GGCTGTATTGCCTCCTAC3'-3', reverse 5'-CCAGTTGGTAAAACATGCGGACTT-3'.

Figure 1. Chemical structure of arbutin.
Effects of arbutin on the mRNA expression levels of COL1A1, β-catenin, RUNX2, BGLAP and SP7. The mRNA expression levels of COL1A1, β-catenin, RUNX2, BGLAP and SP7 were assessed in MC3T3-E1 cells using RT-qPCR following treatment with arbutin at various concentrations (0, 10, 50 or 100 µM) for 3 days. COL1A1 and β-catenin expression levels were increased in osteoblasts treated with 10, 50 and 100 µM arbutin compared with untreated cells (Fig. 4B and C). The expression levels of RUNX2, BGLAP and SP7 were significantly increased following treatment with arbutin at 50 and 100 µM (Fig. 4D-F).

Effects of arbutin on protein expression levels of β-catenin and RUNX2. To investigate the underlying mechanisms of arbutin-induced osteoblast differentiation, the protein expression levels of RUNX2 and β-catenin were examined in MC3T3-E1 cells by western blotting (Fig. 5A and B). Treatment with arbutin at 100 µM significantly increased the protein expression levels of β-catenin and RUNX2 in osteoblasts compared with controls (Fig. 5C and D, respectively). The present results suggested that arbutin may affect osteoblast differentiation by regulating the protein expression levels of β-catenin and RUNX2. To investigate whether arbutin stimulates osteoblastic differentiation via the Wnt/β-catenin signaling pathway, MC3T3-E1 cells were treated with 0.5 µg/ml DKK1 for 6 h prior to treatment with 100 µM arbutin. DKK1 significantly inhibited arbutin-induced RUNX2 protein expression (Fig. 5E). The present results suggested that arbutin may affect osteoblast differentiation via the Wnt/β-catenin signaling pathway.

Discussion
Osteoporosis may result in serious fractures and disabilities, and represents an age-associated problem worldwide (19).
accumulating evidence demonstrated that an imbalance between osteoclasts and osteoblasts in bone formation and resorption may lead to osteoporosis (20). notably, bone formation depends on osteoblast proliferation and differentiation (21,22).

Multiple available drugs used to treat osteoporosis are antiresorptive medications (23); however, these treatments are not able to reverse bone loss (24). Anabolic agents that stimulate bone formation may restore severely damaged skeletal microstructure and loss of bone mass (24). Teriparatide is one anabolic agent that was demonstrated to stimulate bone formation, and clinical trials demonstrated that treatment with teriparatide decreased the risk of new vertebral fractures and increased bone mineral density in the hip, lumbar spine and femoral neck (25). notably, teriparatide is an expensive treatment. Therefore, it is important to develop novel drugs that are able to effectively promote bone formation.

Arbutin is a cytoprotective agent and does not exhibit significant cytotoxic effects at high concentrations (26). although the blood concentration of arbutin is unclear (13), arbutin is used as a urinary antimicrobial medicine and is considered as a safe oral agent (27,28). Previous studies demonstrated that arbutin may exhibit multiple activities, including skin whitening (10,11), anti-inflammatory (29), anticancer (30) and suppression of osteoclast differentiation (13). However, further studies are required to investigate the effects of arbutin on osteoblasts and its potential to be used as a novel compound for the treatment of osteoporosis. Therefore, the present study investigated the effects of arbutin on the proliferation and differentiation of MC3T3-E1 cells and the mechanisms underlying arbutin function in vitro.

Bone formation is related to the number of osteoblasts and the activity of single osteoblasts (31). The number of osteoblasts can be increased by promoting pre-osteoblast replication or differentiation, or by reducing cell death of mature osteoblasts (32). In the present study, arbutin increased proliferation of MC3T3-E1 cells without affecting the apoptotic rate. In addition, arbutin increased cell cycle progression by shifting cells from G1-phase to S-phase. These results suggested that arbutin may induce osteoblastic proliferation.

ALP is an early marker of osteogenic differentiation (33). ALP is associated with calcification of the skeleton during bone formation (34). In the present study, ALP staining results suggested that osteoblasts treated with arbutin at 10, 50 and 100 µM, and with osteogenic supplement for 9 days exhibited an increased activity of ALP, suggesting that arbutin may promote the early differentiation of osteoblasts.
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Figure 4. Effects of arbutin on osteogenic differentiation. (A-F) MC3T3-E1 mouse osteoblast precursor cells were cultured in differentiation medium and treated with 0 (control), 10, 50 or 100 µM arbutin for (A) 9 or (B-F) 3 days. (A) ALP-positive cells are stained in blue or purple. The effects of arbutin on the mRNA expression levels of (B) COL1A1, (C) β-catenin, (D) RUNX2, (E) BGLAP and (F) SP7 in MC3T3-E1 cells. mRNA expression levels were assessed by reverse transcription-quantitative polymerase chain reaction analysis; β-actin served as the internal control. *P<0.05 and **P<0.01 vs. untreated control. ALP, alkaline phosphatase; COL1A1, collagen type I α 1 chain; BGLAP, bone γ-carboxyglutamate protein; SP7, Sp7 transcription factor; RUNX2, runt-related transcription factor 2.

Figure 5. Arbutin promotes MC3T3-E1 cell differentiation via the Wnt/β-catenin signaling pathway. (A) Cells were treated with 0 (control), 10, 50 or 100 µM arbutin for 3 days, and the protein expression levels of β-catenin and RUNX2 were detected by western blot analysis. (B) MC3T3-E1 cells were treated with 0.5 µg/ml DKK1 for 6 h prior to treatment with 100 µM arbutin. Protein expression levels of (C) β-catenin and (D) RUNX2, from Part A, were quantified by densitometric analysis. *P<0.05 and **P<0.01 vs. untreated control. (E) Densitometric analysis of RUNX2 following combined treatment with DKK1 and arbutin, from Part B. *P<0.05 vs. untreated control; **P<0.01 vs. arbutin alone. RUNX2, runt-related transcription factor 2; DKK1, dickkopf WNT signaling pathway inhibitor 1; Wnt, wingless/integrated.
Osteogenesis has been demonstrated to be regulated by various transcription factors, including RUNX2 and SP7, and multiple bone-specific matrix proteins, including ALP, BGLAP and COLIA1 (35). RUNX2 and SP7 are important transcription factors involved in osteoblast differentiation during bone formation (36). COLIA1 is an extracellular matrix protein that promotes bone regeneration and osteoblast differentiation (37). BGLAP is a non-collagenous bone matrix protein that regulate bone turnover and bone mineralization (38). The present study identified that arbutin may induce the mRNA expression levels of important osteogenic regulators, including RUNX2, BGLAP, SP7 and COLIA1. Additionally, the expression level of β-catenin was increased. These results suggested that arbutin may promote osteoblastic differentiation through a mechanism involving Wnt/β-catenin signaling.

The Wnt signaling pathway influences bone formation during development, and bone remodeling during tissue renewal (39). The canonical Wnt signaling pathway was identified to be initiated by Wnt ligand signaling at the cell surface through low-density lipoprotein receptor-related protein 5 or 6 (Lrp5/6) and seven-pass transmembrane Frizzled receptor (40). The interaction between Wnt ligands and their receptor may inhibit GSK3-β in the cytoplasm, leading to the release of β-catenin, the transcriptional mediator of canonical Wnt signaling. Following release, β-catenin is able to enter the nucleus, thus controlling the expression levels of its target genes (41). RUNX2 belongs to the Runt domain gene family and is a transcription factor involved in osteoblastic differentiation (42). RUNX2 serves an important role in coordinating multiple signaling pathways during osteoblast differentiation (43). A previous study demonstrated that canonical Wnt signaling may directly regulate RUNX2. Specifically, the β-catenin/HNF1 homeobox A complex may activate the expression level of RUNX2, thus promoting bone formation (44). DKK1 is a powerful inhibitor of the Wnt/β-catenin canonical pathway, by binding to Lrp5/6 (45,46). The results of the present study suggested that treatment with arbutin significantly increased the protein expression levels of RUNX2 and β-catenin in MC3T3-E1 cells, and DKK1 significantly decreased the protein expression level of RUNX2. The present results suggested that arbutin may promote MC3T3-E1 cell differentiation via the canonical Wnt/β-catenin pathway.

Collectively, to the best of the authors' knowledge, the present study is the first to indicate that arbutin may stimulate proliferation and differentiation of MC3T3-E1 cells via the Wnt/β-catenin signaling pathway. Therefore, arbutin may represent a novel potential candidate for osteoporosis treatment. However, further studies are required to identify the specific role of the Wnt/β-catenin signaling pathway in arbutin-induced osteogenesis, including the phosphorylation of β-catenin at Ser675 (47); further information regarding the role of Wnt/β-catenin signaling may be achieved using Wnt agonists. In the future, further studies may investigate the ability of arbutin to promote bone formation in vivo.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QF, XM and LiY conceived and designed the experiments. XM performed the experiments and wrote the manuscript. SL, LiY, LeY and ML analyzed the data and critically revised the manuscript. QF supervised all research and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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