

Echinacoside suppresses dexamethasone-induced growth inhibition and apoptosis in osteoblastic MC3T3-E1 cells

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Abstract. Glucocorticoids (GCs) are widely used to treat chronic diseases. Prolonged and/or overdose administration of GCs has many side-effects to human health including GC-induced osteoporosis (GIOP). In this investigation, the objective was to assess the influence that echinacoside (ECH) exerts upon dexamethasone-treated murine osteoblastic MC3T3-E1 cells. We found that ECH (5, 10, 20 and 40 mg/l) inhibited dexamethasone (1,000 nM)-suppressed cell viability as demonstrated by Cell Counting Kit-8 (CCK-8) assay. The dose of 10 mg/l was selected for the following experiments because this dose had a better effect than the dose of 5 mg/l, and the doses >10 mg/l had a similar effect as this dose. ECH (10 mg/l) or pifithrin- α (PFT- α) (a p53 inhibitor, 20 μ M) suppressed dexamethasone-induced MC3T3-E1 apoptosis as illustrated by Annexin V/propidium iodide (PI) double-labeling flow cytometry analysis. ECH or PFT- α treatment also alleviated dexamethasone's action of inhibiting Bcl-2 expression as well as dexamethasone's action of stimulating on the expression of p53 and Bax. Moreover, lentivirus mediated-p53 overexpression reversed the effects of ECH in dexamethasone-treated MC3T3-E1 cells, suggesting that ECH induced anti-apoptotic effects in dexamethasone-treated osteoblasts via p53-dependent pathway. In summary, ECH has a protective effect against osteoblastic cell apoptosis induced by dexamethasone, suggesting that ECH may have potentials for clinical application in the treatment of GIOP.

Introduction

Glucocorticoids (GCs) are frequently used to treat a wide spectrum of chronic illnesses, including inflammation, cancer,

and autoimmune disorders (1). However, prolonged and/or overdose administration of GCs has many side-effects, such as hypertension, hyperglycemia, glaucoma, osteonecrosis and osteoporosis (2-4). It is clear that GC use increases bone loss and fracture risk (3-6). GCs can induce apoptosis of osteoblasts and impair the function of these cells. For instance, dexamethasone, a GC hormone, can upregulate the expression of p53 in osteoblastic MC3T3-E1 cells, thus causing cell apoptosis (7,8). Osteoblast apoptosis leads to decreased bone formation. Oral use of bisphosphonates (alendronate and risdrionate) (9,10) has been introduced for the treatment of GC-induced osteoporosis (GIOP). However, low adherence rates limit the efficiency of oral bisphosphonates. Other medications also have limitations such as inconvenient administration [zoledronic acid (11)] and high cost [PTH 1-34 (teriparatide) (12,13)]. Consequently, there is a need for the development of new treatment options for GIOP.

Echinacoside (ECH) is a phenylethanoid glycoside isolated from the traditional Chinese medicine *Herba Cistanches* (14). ECH has protective effects on the neuron system (15-20), liver (21,22) and lung (23) through promoting cell proliferation, and inhibiting inflammatory response, reactive oxygen species (ROS) production and cell apoptosis. Although ECH can also promote osteoblastic bone regeneration and has an extraordinary antiosteoporotic activity in rat model (24,25), there are no existing records describing the effects of ECH on GC-induced osteoblastic cell apoptosis. Thus, we investigated the influence that ECH exerts upon dexamethasone-induced osteoblastic cell apoptosis and elucidated the preliminary mechanism in MC3T3-E1 cells.

Materials and methods

Cultivation of cells. The Cell Bank of the Chinese Academy of Sciences (Shanghai, China) provided the murine osteoblastic MC3T3-E1 cells. Growth of cells took place in α -minimal essential medium (α -MEM) (Hyclone; GE Healthcare, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA) and penicillin/streptomycin (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). Maintenance of cells was conducted in an environment with 95% air and 5% carbon dioxide at a temperature of 37°C.

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The production of lentivirus overexpressing p53. To create p53 expression construct, the protein coding sequence (CDS) region of murine p53 gene was synthesized and inserted into *EcoRI/BamHI* restriction sites of the lentiviral expression vector pLVX-puro (Clontech Laboratories, Inc., Palo Alto, CA, USA) by Genewiz, Inc. (Beijing, China). The construct was verified by DNA sequencing. 293T cells (Shanghai GeneChem Co., Ltd., Shanghai, China) were transfected with a mixture of plasmids, including viral packaging plasmids and p53 expression plasmid (pLVX-p53) or control plasmid (pLVX) via Lipofectamine 2000 (Invitrogen: Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) based on the manufacturer's instructions. The viral supernatant was collected at 48 h after transfection and used to infect MC3T3-E1 cells. Evaluation of p53 expression was conducted at 48 h after viral infection.

Quantitative polymerase chain reaction (qPCR). We used TRIzol reagent (Invitrogen: Thermo Fisher Scientific, Inc.) to separate total RNA and MC3T3-E1 cells, and RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) according to the manufacturer's instructions to reverse-transcribe total RNA. p53 mRNA level was detected via qPCR on ABI Prism 7300 Sequence Detection System (Applied Biosystems: Thermo Fisher Scientific, Inc., Foster City, CA, USA). The primers were as follows: 5'-CCC CTGTCATCTTTTGTCCCT-3' and 5'-AGCTGGCAGAAT AGCTTATTGAG-3' for p53, 5'-CTGCCAGAACATCA TCC-3' and 5'-CTCAGATGCCTGCTTCAC-3' for GAPDH. Then, we assessed p53 mRNA levels via $2^{-\Delta\Delta CT}$ method (26) with GAPDH as internal control.

Western blotting. Lysis of MC3T3-E1 cells took place in radio immunoprecipitation assay buffer with protease inhibitors (Beijing Solarbio Science & Technology Co., Ltd.), and centrifugation of lysates continued for 15 min at a temperature of 4°C at 9,600 x g so that precipitation can be removed. Subsequently, we assessed the amount of protein with the BCA assay kit (Thermo Fisher Scientific, Inc.). Equal amount of protein was added onto a 10 or 15% SDS-PAGE and transferred to a nitrocellulose membrane (EMD Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk and probed with primary antibodies. After washing 3 times with phosphate-buffered saline (PBS), incubation of membranes with horseradish peroxidase conjugated secondary antibody (1:1,000; cat no. A0208; Beyotime Institute of Biotechnology, Shanghai, China) was conducted. We conducted western blotting tests via an enhanced chemiluminescence (ECL) kit (EMD Millipore). GAPDH was used as the internal standard. The sources of primary antibodies were as follows: Bax (Sc-493) and Bcl-2 (Sc-492) both from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); p53 (no. 2524) and GAPDH (no. 5174) both from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Experimental grouping. To determine the suitable dose of dexamethasone, treatment of MC3T3-E1 cells was conducted with a series amounts of dexamethasone (0, 1, 10, 100 and 1,000 nM; Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA). At 48 h after incubating cells, Cell Counting Kit-8 (CCK-8) assays were conducted to detect cell proliferation.

To select the appropriate dose of ECH, treatment of MC3T3-E1 cells was conducted with 1,000 nM of dexamethasone and various doses of ECH (0, 2.5, 5, 10, 20 and 40 mg/l; Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China). Cells grown under normal condition (mock) were served as control. CCK-8 assay was performed after 48 h of culture.

For all other experiments, the MC3T3-E1 cells were split into five groups: group 1 (mock), cells were cultured under normal condition; group 2, cells were transduced with pLVX lentivirus; group 3, cells cultured with 10 mg/l of ECH; group 4, cells with 20 μ M of pifithrin- α (PFT- α ; Selleck Chemicals, Houston, TX, USA); group 5, cells transduced with pLVX-p53 lentivirus and treated with 10 mg/l of ECH. Cells in groups 2-5 were treated with 1,000 nM dexamethasone. Cell apoptosis and protein expression were measured after 48 h of culture.

Cell proliferation assay. We seeded MC3T3-E1 cells into 96-well plates at 3×10^3 cells/well and cultured at normal conditions overnight. Cells were treated as indicated. After 48 h of culture, CCK-8 (Signalway Antibody LLC, College Park, MD, USA) assay was then performed according to the manufacturer's instructions. Briefly, the culture medium in each well was replaced with 100 μ l of 10% CCK-8 solution in medium. After incubating the cells for a duration of 1 h at a temperature of 37°C, we assessed the absorbance at a wavelength of 450 nm with a microplate reader. The relative cell viability (%) with connections to control wells was calculated.

Cell apoptosis evaluation. We used Annexin V-FITC cell apoptosis kit (Beyotime Institute of Biotechnology) for quantifying cells which are undergoing early apoptosis following the manufacturer's instructions. In brief, collection of treated cells was conducted via trypsinization. The cells were washed via PBS, and resuspended with Annexin binding buffer. Incubation of cells was conducted via Annexin V-FITC for a duration of 15 min at a temperature of 4°C followed by incubation with propidium iodide (PI) for 5 min at 4°C. Cells in the lower right quadrant (Annexin V-FITC positive and PI negative) were considered actively undergoing early apoptosis.

Statistical analysis. Data are the means \pm SD for all three independent experiments. Tukey's multiple comparisons test and one-way analysis of variance were conducted to analyze the differences between groups via GraphPad Prism software (version 6.0; GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of the suitable dose of dexamethasone. To determine the suitable dose of dexamethasone, the MC3T3-E1 cells were exposed to increasing amounts of dexamethasone (0, 1, 10, 100 and 1,000 nM) for 48 h. As shown in Fig. 1, relative cell viability as determined by CCK-8 assay was increased in cells treated with low concentrations of dexamethasone (1 and 10 nM), and significantly reduced in cells exposed to high concentrations of dexamethasone (100 and 1,000 nM).

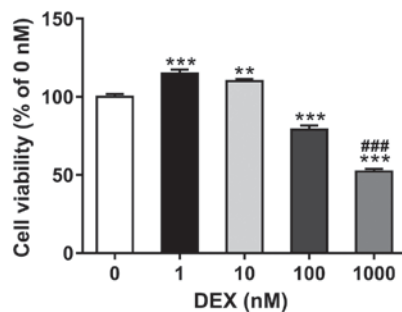


Figure 1. Establishment of the suitable dose of DEX. Concentrations of 0, 1, 10, 100 or 1,000 nM of DEX were added to MC3T3-E1 cells for 48 h, and CCK-8 was then performed. The relative cell viability (%) related to control wells (DEX, 0 nM) was calculated. ** $P < 0.01$ and *** $P < 0.001$ vs. DEX (0 nM); ### $P < 0.001$ vs. DEX (100 nM). Data are the means \pm SD for all three independent experiments, each of which was repeated 3 times. DEX, dexamethasone; CCK-8, Cell Counting Kit-8.

The most significant reduction was observed in cells with 1,000 nM of dexamethasone. Thus, 1,000 nM of dexamethasone was selected in the subsequent assays.

Effect of ECH on dexamethasone-induced cell growth inhibition. We then explored the effect of ECH on dexamethasone-induced cell growth inhibition. Treatment of MC3T3-E1 cells was conducted with 1,000 nM of dexamethasone and various doses of ECH (0, 2.5, 5, 10, 20 and 40 mg/l; Fig. 2). At 48 h post-treatment, the addition of ECH at doses of 5, 10, 20 and 40 mg/l notably decreased dexamethasone-induced cell damage. ECH at a dose of 10 mg/l has more obvious effect than that at a dose of 5 mg/l. Cell viability was similar in cells treated with 10, 20 and 40 mg/l of ECH. Therefore, 10 mg/l of ECH was used in the following experiments.

Overexpression of p53 by constructing lentivirus. Dexamethasone can upregulate p53, thereby inducing osteoblasts apoptosis (8). To study the effects of ECH on dexamethasone-induced p53 expression, we constructed lentivirus encoding p53 (pLVX-p53) and transduced the virus into MC3T3-E1 cells. The expression of p53 was analyzed at 48 h after transduction. Fig. 3 shows that p53 mRNA and protein levels were increased to >2 times in pLVX-p53-transduced cells as compared to cells with control virus (pLVX).

p53 was involved in the effect of ECH on dexamethasone-induced apoptosis. The MC3T3-E1 cells were treated with pLVX or pLVX-p53, 10 mg/l of ECH, 20 μ M of PFT- α (a p53 inhibitor), and 1,000 nM of dexamethasone, and then cell apoptosis was measured via Annexin V-FITC apoptosis kit. As shown in Fig. 4A, dexamethasone treatment remarkably induced cell apoptosis in comparison to the cells cultured under normal condition (mock). ECH and PFT- α treatment significantly protected the MC3T3-E1 cells against dexamethasone-induced apoptosis. Additionally, p53 overexpression reversed the protective effects of ECH.

Bcl-2 and Bax, as important apoptosis regulators, are known to be regulated by p53 (27). Western blotting indicated that the changes of p53 and Bax protein expression were consistent with the alterations of cell apoptosis, whereas Bcl-2 expression changed in the opposite direction (Fig. 4B and C).

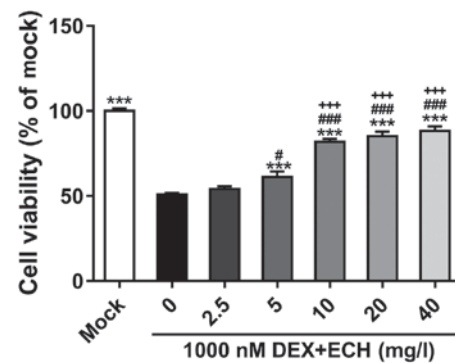


Figure 2. Effect of ECH on DEX-induced cell growth inhibition. MC3T3-E1 cell exposure to various amounts of ECH (0, 2.5, 5, 10, 20 and 40 mg/l) in the presence of 1,000 nM of DEX for 48 h, and CCK-8 was then performed. Cells grown under normal condition (mock) served as negative control. *** $P < 0.001$ vs. 1,000 nM DEX + 0 mg/l ECH; * $P < 0.05$ and ### $P < 0.001$ vs. 1,000 nM DEX + 2.5 mg/l ECH; *** $P < 0.001$ vs. DEX + 1 μ M vs. 1,000 nM DEX + 5 mg/l ECH. Data are the means \pm SD for all three independent experiments, each of which was repeated 3 times. ECH, echinacoside; DEX, dexamethasone; CCK-8, Cell Counting Kit-8.

Notably, the protein levels of p53 were significantly suppressed by ECH and PFT- α treatment in cells overexpressing p53 and treated with dexamethasone (Fig. 4C). These data suggest that ECH might exert protective effects on dexamethasone-induced apoptosis via inhibiting p53 expression.

Discussion

The anti-apoptosis function of ECH has been described in acute liver injury model induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS), and in human neuroblastoma (SH-SY5Y) cells induced by tumor necrosis factor- α or 1-methyl-4-phenylpyridinium ion (MPP $^{+}$) (17,22,28). It has been reported that dexamethasone treatment can inhibit the proliferation and induce MC3T3-E1 cell apoptosis (8,29). In the current study, relative cell viability was significantly reduced in cells exposed to high concentrations of dexamethasone (100 and 1,000 nM), which was consistent with a previous study (8). Additional ECH treatment (5-40 mg/l) remarkably inhibited dexamethasone-suppressed cell viability. Moreover, dexamethasone exposure (1,000 nM) stimulated MC3T3-E1 cell apoptosis, which the ECH treatment restrained to a significant extent. The report gave a first depiction of how ECH is protected against dexamethasone-induced osteoblast cell apoptosis and suggested that ECH may have potential for clinical application in GIOP.

Furthermore, we tried to explore the preliminary mechanism of the protective effects of ECH. Dexamethasone can enhance p53 transcription, thereby inducing osteoblasts apoptosis and cell cycle arrest (8). p53 is downregulated by ECH in SH-SY5Y cells (28). Here, PFT- α (a p53 inhibitor) had similar protective effects as ECH, while p53 overexpression reversed the protective effects of ECH. The changes of p53 protein expression under different conditions were in agreement with the alterations of cell apoptosis. These results indicate that ECH might exert protective effects on dexamethasone-induced osteoblastic cell apoptosis via inhibiting p53 expression. Bcl-2 family proteins, such as Bcl-2 and Bax, can alter the permeability of

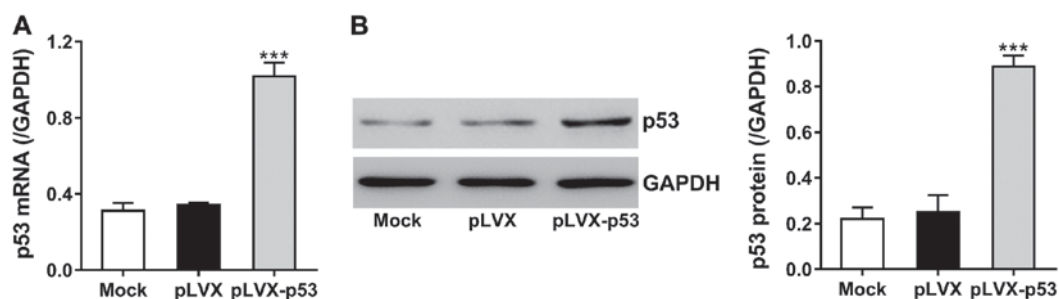


Figure 3. Overexpression of p53 by constructing lentivirus. The protein CDS region of murine p53 gene was inserted into the lentiviral expression vector pLVX-puro. Lentivirus encoding p53 (pLVX-p53) and control virus (pLVX) were packaged in 293T cells and used to infect MC3T3-E1 cells. We evaluated (A) mRNA levels of p53 with qPCR and (B) protein levels of p53 with western blotting, at 48 h after viral infection. *** $P < 0.001$ vs. pLVX. CDS, coding sequence; PCR, polymerase chain reaction.

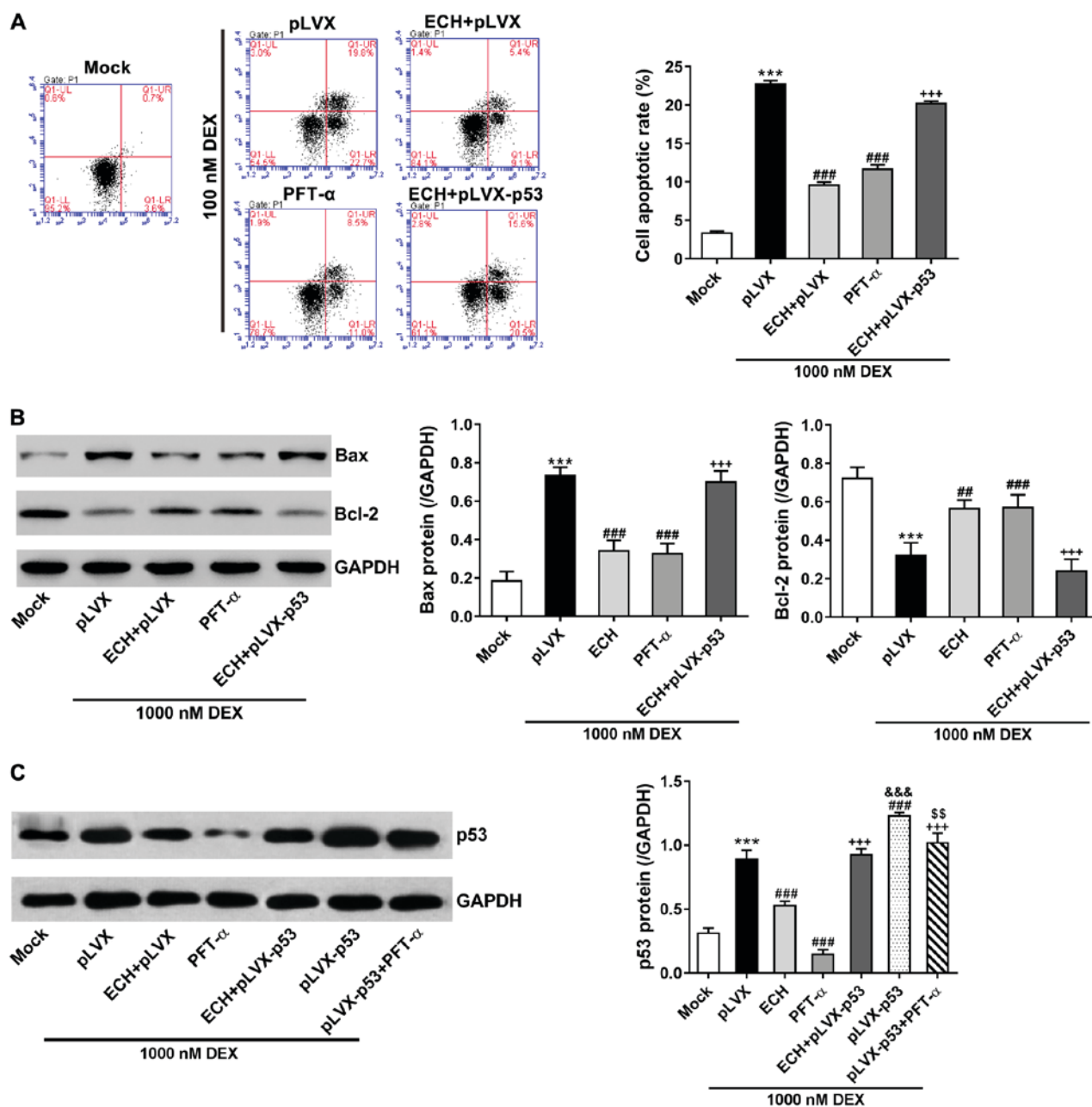


Figure 4. p53 was involved in the effect of ECH on DEX-induced apoptosis. The MC3T3-E1 cells were treated with pLVX or pLVX-p53, 10 mg/l of ECH, 20 μ M of PFT- α , and 1,000 nM of DEX as indicated. Cells without any treatment (mock) served as negative control. (A) At 48 h after treatment, we assessed cell apoptosis via Annexin V-FITC apoptosis kit. (B and C) At 48 h after treatment, we analyzed protein levels of p53, Bax and Bcl-2 via western blotting with GAPDH as loading control. *** $P < 0.001$ vs. mock; ## $P < 0.01$ and ### $P < 0.001$ vs. pLVX + 1,000 nM DEX; +++ $P < 0.001$ vs. ECH; &&& $P < 0.001$ vs. ECH + pLVX-p53 + 1,000 nM DEX; ++ $P < 0.01$ vs. PFT- α + 1,000 nM DEX. ECH, echinacoside; DEX, dexamethasone; PFT- α , pifithrin- α .

mitochondrial membrane, activate effector caspases and cause cell apoptosis (30). It is well-known that p53 can regulate Bcl-2 and Bax (27). Here, treatment with PFT- α or ECH attenuated the inhibitory effects of dexamethasone on anti-apoptosis protein Bcl-2 levels as well as the stimulatory effects dexamethasone has on pro-apoptosis protein Bax levels, while p53 overexpression reversed the effects of ECH. These results further demonstrated the involvement of p53 in the protective function of ECH.

In summary, we demonstrated that ECH has a promising protective effect on osteoblastic cell apoptosis induced by dexamethasone, suggesting that ECH may have potential for clinical application in the treatment of GIOP.

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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

SL performed qPCR. SL and HJ were responsible for western blotting. XG helped with cell proliferation and apoptosis evaluation. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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