

Parthenolide inhibits hydrogen peroxide-induced osteoblast apoptosis

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Abstract. Parthenolide is a natural product from the shoots of *Tanacetum parthenium* that has been demonstrated to have immunomodulatory effects in a number of diseases. The present study aimed to determine the effect and mechanism of parthenolide on the apoptotic ability of H₂O₂-induced osteoblasts. Cell viability was analyzed with a MTT assay and the apoptotic rate was subsequently measured using flow cytometry. The activity of the antioxidative enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX), and the serum marker enzymes alkaline phosphatase (ALP), malondialdehyde (MDA) and lactate dehydrogenase (LDH) was measured. Reverse transcription-quantitative polymerase chain reaction and western blot analyses were performed to analyze the expression levels of osteogenesis and oxidative stress-associated genes. The results indicated that parthenolide increased cell viability and inhibited the apoptosis of H₂O₂-induced osteoblasts. Parthenolide decreased the levels of reactive oxygen species, MDA, LDH and ALP. SOD and GPX levels were increased by parthenolide in H₂O₂-induced osteoblasts. This suggested that parthenolide may break the equilibrium state of oxidative stress and inhibit cellular apoptosis. Parthenolide additionally increased the expression levels of oxidative stress-associated genes, including nuclear factor erythroid 2 like 2, hemeoxygenase-1 and quinone oxidoreductase 1 in H₂O₂-induced osteoblasts. Furthermore, parthenolide increased the expression of osteogenesis-associated genes, including runt-related transcription factor 2, osteopontin, osteocalcin and collagen 1 in H₂O₂-induced osteoblasts. Therefore, it was concluded that parthenolide may be used in the treatment of osteoporosis.

Introduction

Osteoporosis is among the most common health problems in the elderly (1). Normal calcium homeostasis and supplementation are associated with good bone health (2). However, it has been demonstrated that calcium supplementation may cause a variety of adverse conditions, including cardiovascular disease, constipation and kidney stone development (3,4). Therefore, the discovery of novel osteoporosis therapies is of high importance.

Cellular apoptosis may be induced by a number of factors (5,6). Among the most important factors is oxidative stress (7). Oxidative stress has been confirmed to be associated with osteonecrosis (8,9). Hydrogen peroxide (H₂O₂) is a strong oxidizer which is able to induce cells to produce large amounts of reactive oxygen species (ROS) (10). ROS are produced in mitochondria as normal products of cellular metabolism. Elevated ROS is related to oxidative stress, causing cellular dysfunction and apoptosis (11,12). ROS may damage double-stranded DNA, leading to abnormal variation (13,14) and may additionally induce oxidative stress-induced apoptosis in tissues (15,16). H₂O₂ is a key metabolite of oxidation reactions and, therefore, has critical involvement in the pathology of diseases mediated by oxidative stress (15,16). Accumulating evidence has indicated that H₂O₂ may regulate cell function and induce cell death (17). H₂O₂ may additionally penetrate the cell membrane and act as a secondary messenger in signal transduction pathways (18). A previous study indicated that the manner of H₂O₂-induced cell death is associated with cell type, H₂O₂ concentration and the type of stimulation received (19).

Parthenolide is a traditional medicine with immunomodulatory effects (20). It has additionally been demonstrated to have the potential to treat certain types of cancer, including leukemia, and hepatic and breast cancer (21-24). Parthenolide is able to regulate multiple cellular and molecular signals in order to induce tumor cell apoptosis (25). It may additionally regulate the expression of the apoptosis regulator Bcl-2 (Bcl-2) protein family and caspases (25), and promote the loss of mitochondrial function (26). This evidence highlights the potential use of parthenolide in tumor therapy.

The specific effects of parthenolide on osteoblasts are not completely understood. Therefore, the purpose of the present study was to investigate the effect of parthenolide on osteoblast

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proliferation and apoptosis. First, the effects of parthenolide on osteoblast viability and H₂O₂-induced apoptosis were demonstrated. The influence of parthenolide on the expression of ROS, malondialdehyde (MDA), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), superoxide dismutase (SOD) and glutathione peroxidase (GPX) in H₂O₂-induced osteoblasts was additionally investigated. The regulation of oxidative stress-associated and osteogenesis-associated gene expression by parthenolide was subsequently analyzed. The results of the present study indicated that parthenolide may be used as a therapy for osteoporosis.

Materials and methods

Osteoblast sample. Osteoblasts were acquired from an 8-year-old patient with developmental dislocation of the hip. The operation was performed in department of orthopedics, the Fifth People's Hospital of Yuhang District, Hangzhou, Zhejiang in August, 2016. Informed consent was obtained from the patient and the patient's guardian. The present study was approved by The Ethics Committee of The Fifth People's Hospital of Yuhang District (Hangzhou, China). During the surgery, ~1.5 cm² of the cancellous bone of the iliac crest was removed and placed in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 15% newborn calf serum (Thermo Fisher Scientific, Inc.) in sterile conditions.

Separation and cultivation of osteoblasts. The bone surface-attached connective tissues were eliminated and repeatedly washed with PBS. The bone tissue was cut (1 mm³) and washed with PBS. The bone tissue was subsequently digested with 0.25% trypsin (Thermo Fisher Scientific, Inc.) for 30 min at 37°C, and 0.1% collagenase type II (Thermo Fisher Scientific, Inc.) for 1 h at 37°C. The supernatant was collected and centrifuged at 1,000 x g for 10 min at 4°C. Cell deposits were placed in DMEM/F12 (Thermo Fisher Scientific, Inc.) with 15% newborn calf serum (Thermo Fisher Scientific, Inc.).

Cell treatment. Osteoblasts at a density of 10,000 cells were treated with PBS (control) and increasing concentrations of parthenolide (0, 5, 10, 15, 20, 25 and 30 μM) for 24 and 48 h. Osteoblasts were seeded into 6-well plates at a concentration of 3x10⁵ cells/ml and treated with PBS, 0.8 mmol/l H₂O₂ or 0.8 mmol/l H₂O₂ and parthenolide (5, 10 and 20 μM) for 24 h.

ALP staining. Osteoblasts (50,000 cells/well) were seeded in 24-well plates and induced in a humidified incubator for 24 h at 37°C. ALP activity was detected using the ALP staining kit (Merck KGaA, Darmstadt, Germany), according to the manufacturer's protocol. Osteoblasts were observed under a Wild Heerbrugg M400 Zoom Makroskop (Wild Heerbrugg, Heerbrugg, Switzerland) at x200 magnification.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA was obtained using TRIzol (Thermo Fisher Scientific, Inc.), and RNA was reverse-transcribed to cDNA using a Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) with random primers, according to the manufacturer's protocol.

SYBR-Green PCR Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to detect the mRNA expression levels, and the assay was performed on an ABI 7500 system (Thermo Fisher Scientific, Inc.). Thermocycling conditions were: 95°C for 15 sec; 40 cycles at 95°C for 10 sec, 59°C for 10 sec and 72°C for 15 sec. The relative mRNA expression levels were normalized to GAPDH. The data were analyzed using the 2^{-ΔΔC_t} method (27). The primer sequences were as follows: Bcl-2 forward, 5'-GCCTTCTTTGAGTTCGGTGG-3' and reverse, 5'-GAAATCAAACAGAGGCCGCA-3'; apoptosis regulator BAX (Bax) forward, 5'-GAGCTGCAGAGGATGATTGC-3' and reverse, 5'-CCAATGTCCAGCCCATGATG-3'; runt related transcription factor 2 (Runx2) forward, 5'-CTGTGGTTACTGTTCATGGCG-3' and reverse, 5'-AGGTAGCTACTTGGGGAGGA-3'; osteopontin (OPN) forward, 5'-ACTGATTTTCCCACGGACCT-3' and reverse, 5'-CTCTCGCTTTCCATGTGTG-3'; osteocalcin (OCN) forward, 5'-CTCACACTCCTCGCCCTATT-3' and reverse, 5'-AATCGTTCACAGTCCGGATT-3'; collagen 1 (Col-1) forward, 5'-TCATTCCGCAAACCCACTTG-3' and reverse, 5'-CCC CAATCGAGAAGCCATTG-3'; nuclear factor erythroid 2 like 2 (Nrf2) forward, 5'-GGTTGCCACATTCCCAAAT-3' and reverse, 5'-AGCAATGAAGACTGGGCTCT-3'; heme oxygenase 1 (HO1) forward, 5'-TTCAGAAGGGTCAGGTGTCC-3' and reverse, 5'-CAGTGAGGCCCATACCAGAA-3'; NAD (P) H quinone oxidoreductase 1 (NQO1) forward, 5'-CCTTCCGGAGTAAGAAGGCA-3' and reverse, 5'-TCTCCAGGCGTTTCTTCCAT-3'; and GAPDH forward, 5'-GCCATCACAGCAACACAGAA-3' and reverse, 5'-GCCATACCA GTAAGCTTGCC-3'.

Western blot analysis. Total protein was extracted using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentration was measured using the Pierce Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.) and detected using a protein reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (30 μg) were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (PerkinElmer Inc., Waltham, MA, USA). The membranes were blocked with 5% low-fat milk (BD Biosciences, Franklin Lakes, NJ, USA) for 2 h at room temperature and subsequently incubated with the following primary antibodies overnight at 4°C: Anti-GAPDH (1:2,000; cat. no. ab8245; Abcam, Cambridge, UK), anti-Bax (1:1,000; cat. no. ab32503; Abcam), anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam), anti-Runx2 (1:1,500; cat. no. ab23981; Abcam), anti-OPN (1:1,500; cat. no. ab166709; Abcam), anti-OCN (1:1,500; cat. no. ab93876; Abcam), anti-Col-1 (1:1,000; cat. no. ab6308; Abcam), anti-Nrf2 (1:1,000; cat. no. ab62352; Abcam), anti-HO1 (1:1,200; cat. no. ab69544; Abcam) and anti-NQO1 (1:1,000; cat. no. ab28947; Abcam). The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,000; cat. no. ab97165; Abcam) for 2 h at room temperature. The protein expression levels were detected using the enhanced chemiluminescence substrate kit (Thermo Fisher Scientific, Inc.) and the enhanced chemiluminescence detection system (GE Healthcare, Chicago, IL, USA). The densitometric analysis

was performed by Labworks Software (version 4.5; UVP, Inc., Upland CA, USA).

ROS, SOD, MDA, GPX and LDH activity detection. Osteoblasts were treated with PBS, 0.8 mmol/l H_2O_2 or 0.8 mmol/l H_2O_2 and parthenolide (5, 10 and 20 μ M) for 12, 24 and 48 h. The fluorescent dye dihydroethidium (DHE; cat. 309800; Merck KGaA, Darmstadt, Germany) was used to measure ROS activity, as described previously (28). Treated cells were incubated with 2.5 mmol/l DHE for 25 min at 37°C. Cells were subsequently washed with PBS, digested with 0.25% trypsin (Sigma-Aldrich; Merck KGaA) and incubated with DHE. The results were detected by using flow cytometry equipped with an argon laser and Cell Quest™ software (version 3.3; BD Biosciences, Franklin Lakes, NJ, USA). The experiment was repeated at least three times. SOD and MDA activity was detected using a commercial kit (Sigma-Aldrich; Merck KGaA) (29) and a MDA activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the manufacturers' protocols. LDH activity was detected using a diagnostic kit (cat. no. GL2623; Randox Laboratories Ltd., Crumlin, UK), according to the method of Cabaud and Wroblewski (30). GPX enzyme activity was detected using the cellular GPX assay kit (Sigma-Aldrich; Merck KGaA) as described previously (31). The colorimetric reactions of LDH and GPX were determined using a microplate spectrophotometer system (BioRad-680, Bio-Rad Laboratories, Inc.) at 440 and 412 nm, respectively.

ELISA analysis. Osteoblasts were treated with PBS (control), 0.8 mmol/l H_2O_2 or 0.8 mmol/l H_2O_2 and parthenolide (5, 10 and 20 μ M) for 12, 24 and 48 h. Treated osteoblasts were subsequently washed with PBS and centrifuged at 1,000 x g for 5 min at 4°C. The culture supernatant (500 μ l) was harvested and ALP activity was measured using an ELISA kit (cat. AR001, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. A 96-well plate was incubated with anti-ALP (1:1,000, cat. no. ab83259; Abcam) for 1 h at 37°C and subsequently incubated with HRP-conjugated secondary antibody (1:5,000; cat. no. ab97165; Abcam) for 1 h at 37°C. The absorbance was measured at 450 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Inc.).

Cell viability assay. Treated osteoblasts (2×10^3 cells/well) were seeded into a 96-well plate and maintained at 37°C for 12, 24 and 48 h. At the indicated time-points (12, 24 and 48 h), 15 μ l MTT solution (5 mg/ml) was added into each well, respectively. Following incubation for 4 h, cells were treated with 150 μ l dimethylsulfoxide. The absorbance was measured using a microplate reader at 490 nm.

Flow cytometer analysis. Osteoblasts (1×10^6 cells/well) were seeded into 6-well plates and treated with 0.8 mmol/l H_2O_2 or 0.8 mmol/l H_2O_2 and parthenolide (5, 10 and 20 μ M) for 24 h. Treated cells were washed with PBS and centrifuged at 1,000 x g for 5 min at 4°C. The precipitate was re-suspended in 0.5 ml binding buffer, including 5 μ l annexin V-fluorescein isothiocyanate and propidium iodide (BD Biosciences) for

20 min in the dark. The apoptotic rate was examined using a FACSCalibur flow cytometer (BD Biosciences) with CXP Analysis software version 2.2 (Beckman Coulter, Brea, CA, USA). Cells in the lower left quadrant of each picture correspond to normal cells (Annexin V/PI). Cells in right lower quadrant correspond to early apoptotic cells (Annexin V+/PI-). Cells in the right upper quadrant correspond to late apoptotic/dead cells (Annexin V+/PI+).

Statistical analysis. Results were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA), and the statistical significance was calculated using Student's paired t-test and one-way and/or multiple-comparison analysis of variance followed by Tukey's test. All data are presented as the mean \pm standard deviation of three repeated experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of parthenolide on osteoblast viability. Under the microscope, control osteoblasts were observed to be triangular, polygonal or irregular. The nuclei were elliptical with one or two nucleoli and black cytoplasmic particles were present. The dark nuclei in the cells were positive for ALP (Fig. 1A). Osteoblasts were treated with different concentrations of parthenolide (0, 5, 10, 15, 20, 25 and 30 μ M) for 24 and 48 h. MTT assays were performed to examine osteoblast viability. The results indicated that parthenolide significantly improved osteoblast viability in a dose- and time-dependent manner (** $P < 0.01$; *** $P < 0.001$; Fig. 1B).

Effect of parthenolide on H_2O_2 -induced osteoblasts. To examine the possible biological functions of parthenolide and H_2O_2 in osteoblasts, osteoblasts were treated with 0.8 mmol/l H_2O_2 or 0.8 mmol/l H_2O_2 and parthenolide (5, 10 and 20 μ M) for 12, 24 and 48 h. An MTT assay was performed to examine osteoblast viability in a H_2O_2 environment. Results indicated that H_2O_2 significantly decreased osteoblast viability, and this decrease was rescued by parthenolide in a dose- and time-dependent manner (*** $P < 0.001$, Fig. 1C).

Parthenolide inhibits apoptosis in H_2O_2 -induced osteoblasts. To further investigate the effect of parthenolide on H_2O_2 -induced osteoblast apoptosis, flow cytometry was performed. H_2O_2 significantly increased the apoptotic rate in osteoblasts, and parthenolide significantly decreased the H_2O_2 -induced increase in osteoblast apoptosis (*** $P < 0.001$ vs. control group; ** $P < 0.01$, *** $P < 0.001$ vs. H_2O_2 group; Fig. 2A and B). To further understand the molecular mechanism of the function of parthenolide, RT-qPCR and western blot analyses were used to analyze Bax and Bcl-2 expression. The results revealed that H_2O_2 significantly increased the expression of Bax, and significantly decreased the expression of Bcl-2. Parthenolide significantly reversed the alterations observed in H_2O_2 -induced osteoblasts (*** $P < 0.001$ vs. control group, *** $P < 0.001$ vs. H_2O_2 group, Fig. 2C-E).

Parthenolide decreases the levels of ROS, MDA, LDH and ALP, and increases SOD and GPX levels in H_2O_2 -induced osteoblasts. Cellular apoptosis and proliferation are two important events

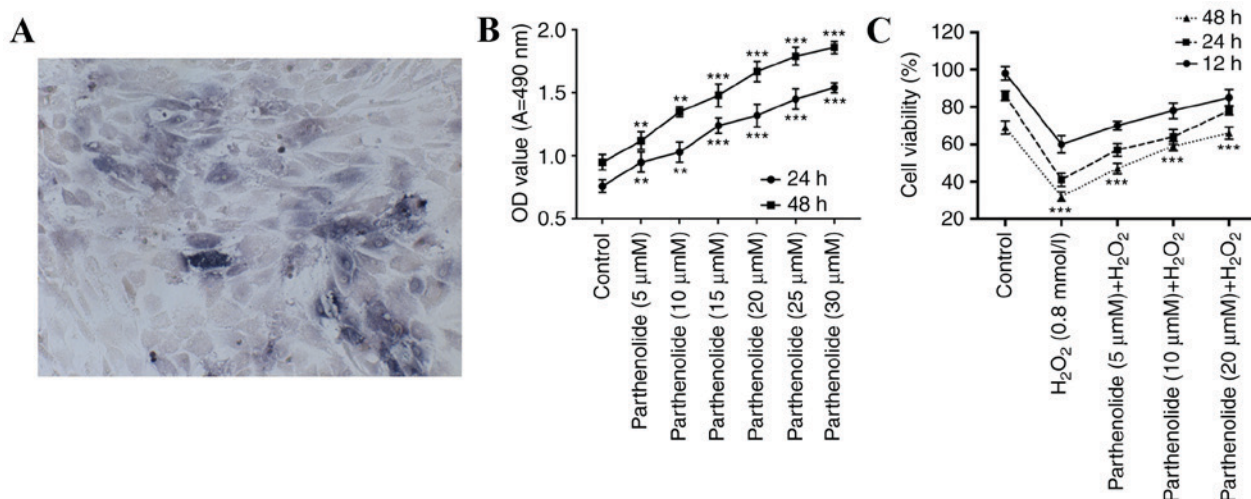


Figure 1. Protective effect of parthenolide on osteoblast cells. (A) Representative image of osteoblasts stained for alkaline phosphatase (magnification, x200). (B) Cell viability was measured by MTT assay in osteoblasts treated with increasing concentrations of parthenolide for 24 and 48 h, and (C) osteoblasts treated with H₂O₂, and increasing concentrations of parthenolide, for 12, 24 and 48 h. **P<0.01, ***P<0.001 vs. control group.

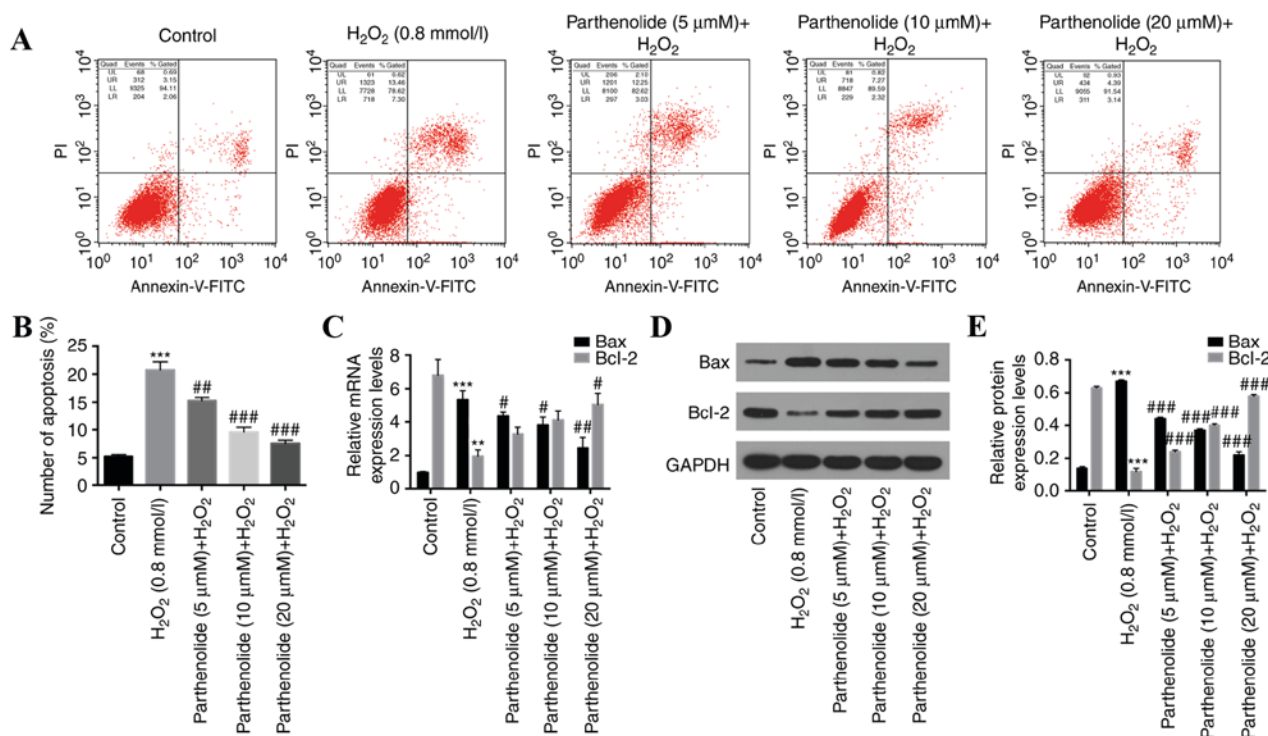


Figure 2. Parthenolide inhibits H₂O₂-induced osteoblast apoptosis. Osteoblasts were treated with 0.8 mmol/l H₂O₂, or 0.8 mmol/l H₂O₂ and increasing concentrations of parthenolide for 24 h. (A) Osteoblast apoptosis was analyzed by flow cytometry and (B) the apoptotic cell number was quantitatively counted. (C) Reverse transcription quantitative-polymerase chain reaction analysis was used to analyze Bax and Bcl-2 mRNA expression levels. (D) Western blot analysis was used to measure Bax and Bcl-2 protein expression levels. (E) Relative quantification of Bax and Bcl-2 protein expression levels. **P<0.01, ***P<0.001 vs. control group. #P<0.05, ##P<0.01, ###P<0.001 vs. H₂O₂ group. Bcl-2, apoptosis regulator Bcl-2; Bax, apoptosis regulator BAX; FITC, fluorescein isothiocyanate; PI, propidium iodide.

affected by oxidative stress (32-35). Numerous studies have demonstrated that ROS levels are closely associated with the induction of apoptosis in a number of pathophysiological conditions (36,37). Oxidative stress may cause an imbalance between ROS and activity of antioxidative enzymes, including SOD and GPX (38). The influence of parthenolide and H₂O₂ on serum marker enzymes MDA, LDH and ALP was additionally investigated (39). Osteoblasts were treated with PBS,

0.8 mmol/l H₂O₂ or 0.8 mmol/l H₂O₂ and parthenolide (5, 10 and 20 μM) for 24 h. The results indicated that H₂O₂ significantly increased ROS (Fig. 3A), MDA (Fig. 3C), LDH (Fig. 3D) and ALP (Fig. 3F) levels. SOD (Fig. 3B) and GPX (Fig. 3E) levels were significantly decreased. Parthenolide significantly reversed the alterations observed in H₂O₂-induced osteoblasts (***P<0.001 vs. control group; #P<0.05, ##P<0.01, ###P<0.001 vs. H₂O₂ group).

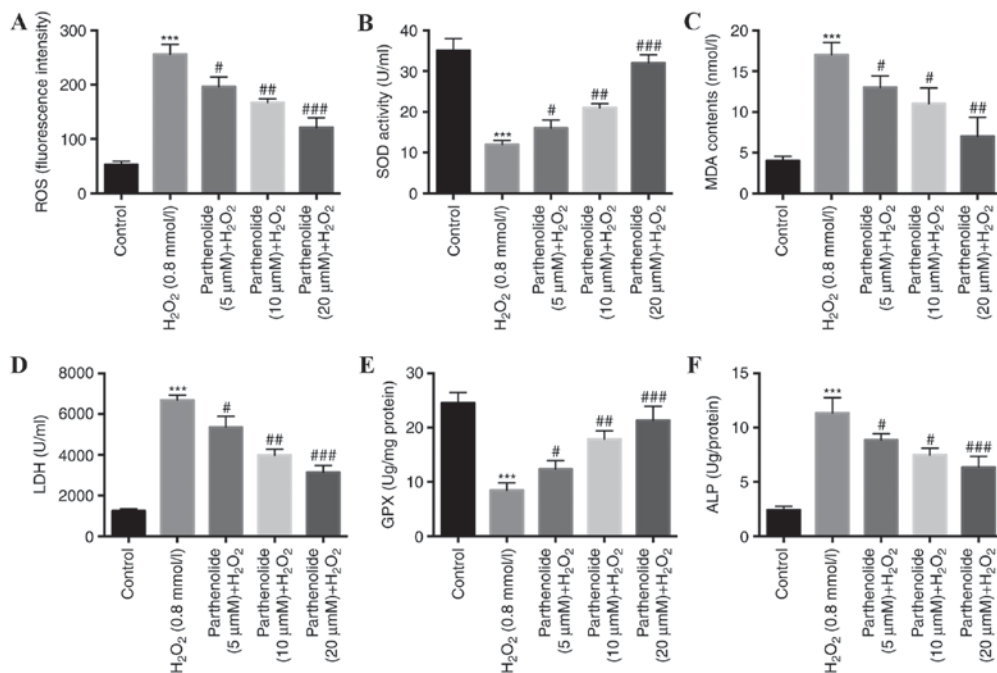


Figure 3. Parthenolide decreased the levels of ROS, MDA, LDH and ALP, and increased the levels of SOD and GPX in H₂O₂-induced osteoblasts. Osteoblasts were treated with 0.8 mmol/l H₂O₂ or 0.8 mmol/l H₂O₂ and parthenolide at increasing concentrations for 24 h (A) ROS fluorescence intensity was detected by flow cytometry with a fluorescence dye DHE. (B) SOD activity was determined with a colorimetric assay. (C) MDA activity was determined with a colorimetric assay. (D) LDH activity was determined with a cytotoxicity assay. (E) GPX activity was determined with a glutathione peroxidase cellular activity assay. (F) ALP activity was determined with an ELISA kit. ***P<0.001 vs. control; **P<0.05, #P<0.01, ##P<0.001 vs. H₂O₂ group. ROS, reactive oxygen species; MDA, malondialdehyde; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; SOD, superoxide dismutase; GPX, glutathione peroxidase.

Parthenolide upregulates osteogenesis-associated genes (Runx2, OPN, OCN, and Col-1) in osteoblasts, mediated by H₂O₂. It has been reported that Runx2, OPN, OCN, and Col-1 are key genes associated with osteogenesis (40). Therefore, the impact of parthenolide and H₂O₂ on the expression of these genes in osteoblasts was investigated. RT-qPCR and western blot analyses were performed to detect Runx2, OPN, OCN and Col-1 expression in treated osteoblasts. H₂O₂ was demonstrated to significantly inhibit Runx2, OPN, OCN, and Col-1 expression. Parthenolide rescued this H₂O₂-mediated inhibition (***P<0.001 vs. control group; #P<0.05, ##P<0.01, ###P<0.001 vs. H₂O₂ group; Fig. 4).

Parthenolide increases Nrf2, HO-1 and NQO1 expression in osteoblasts, mediated by H₂O₂. The expression of Nrf2, HO-1 and NQO1 was measured by RT-qPCR and western blot analyses, to further elucidate the molecular mechanisms of action of parthenolide and H₂O₂ in osteoblasts. H₂O₂ was demonstrated to significantly decrease the expression of Nrf2, HO-1 and NQO1. Parthenolide reversed this decrease (***P<0.001 vs. control group; #P<0.05, ##P<0.01, ###P<0.001 vs. H₂O₂ group; Fig. 5).

Discussion

Large-scale and multi-directional osteoporosis research has made significant progress worldwide. The study of the effect of Chinese medicine in the process of bone collagen formation remains in its infancy, particularly in regards to the underlying molecular mechanisms (41). Tanacetum vulgare is an herb used as a traditional Chinese medicine for its analgesic,

antibacterial and anti-tumor properties; it is used to treat fever, migraine and joint pain (42). Tanacetum vulgare contains a variety of medicinal ingredients, including parthenolide, which is an active component of the sesquiterpene lactones (43). Recent studies have demonstrated that parthenolide has strong anti-tumor activity and may enhance the sensitivity of tumor cells to apoptosis signaling (44). The molecular mechanism of action of parthenolide in osteoporosis is unclear.

Oxidative stress is an inevitable condition in all organisms. A series of adaptive mechanisms exist to protect cells from damage, although various harmful stimuli may cause an imbalance in the equilibrium of oxidation, leading to the induction of apoptosis and even pathological damage (45). Oxidative stress may induce apoptosis through the mitochondrial, death receptor and endoplasmic reticulum stress pathways (46), in addition to through the activation of the protein kinase, nuclear factor-κB and caspase pathways (47). It has been demonstrated that oxidative stress may be a risk factor associated with the development and progression of osteoporosis (48). Increasing evidence additionally suggests that ROS accumulation leads to oxidative stress under conditions of aging and certain illnesses or medicines, contributing to the development and progression of osteoporosis (49).

The present study demonstrated that parthenolide inhibited the decrease in osteoblast viability and the increase in apoptosis mediated by H₂O₂. Parthenolide additionally decreased Bax expression and increased Bcl-2 expression in osteoblasts induced by H₂O₂. The expression of osteogenesis-associated genes Runx2, OPN, OCN and Col-1 was increased in H₂O₂-induced osteoblasts. Additionally, previous studies (38,50) revealed that oxidative stress affects the activity

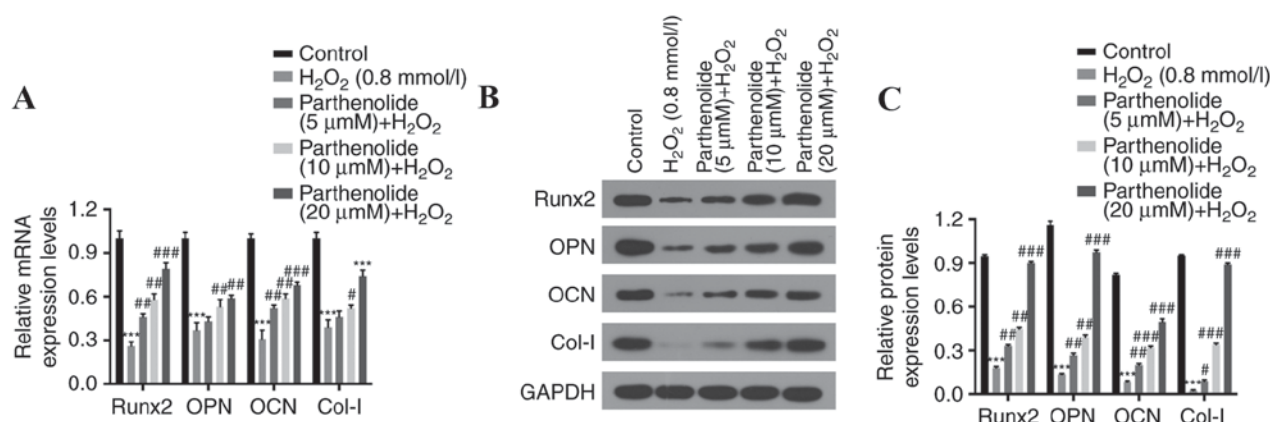


Figure 4. Parthenolide upregulates the osteogenesis-associated genes Runx2, OPN, OCN and Col-1 in H₂O₂-induced osteoblasts. Osteoblasts were treated with 0.8 mmol/l H₂O₂ or 0.8 mmol/l H₂O₂ and increasing concentrations of parthenolide for 24 h. (A) Reverse transcription quantitative-polymerase chain reaction analysis was used to analyze Runx2, OPN, OCN, and Col-1 mRNA expression levels. (B) Protein expression levels were detected by western blot analysis and (C) the relative quantification of proteins was calculated. ***P<0.001 vs. control group; #P<0.05, ##P<0.01, ###P<0.001 vs. H₂O₂ group. Runx2, runt related transcription factor 2; OPN, osteopontin; OCN, osteocalcin; Col-1, collagen 1.

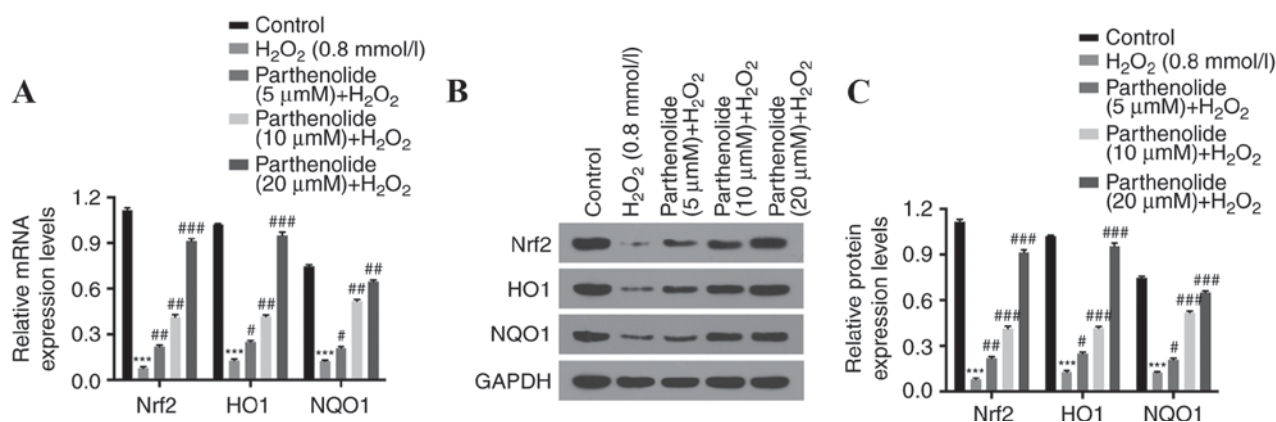


Figure 5. Parthenolide increases Nrf2, HO-1 and NQO1 expression in H₂O₂-induced osteoblasts. Osteoblasts were treated with 0.8 mmol/l H₂O₂ or 0.8 mmol/l H₂O₂ and increasing concentrations of parthenolide for 24 h. (A) Reverse transcription quantitative-polymerase chain reaction analysis was used to analyze Nrf2, HO-1 and NQO1 mRNA expression levels. (B) Protein expression levels were detected by western blot analysis and (C) the relative quantification of proteins was calculated. ***P<0.001 vs. control group; #P<0.05, ##P<0.01, ###P<0.001 vs. H₂O₂ group.

of the antioxidant enzymes SOD and GPX and serum marker enzymes MDA, LDH and ALP (38,50). The present study demonstrated that parthenolide decreased the H₂O₂-induced increase in ROS, MDA, LDH, and ALP levels. SOD and GPX levels were also rescued in H₂O₂-induced osteoblasts.

A previous study indicated that parthenolide, a sesquiterpene lactone obtained from *Tanacetum parthenium*, has high antineoplastic activity (44). Parthenolide has been demonstrated to inhibit the growth and induce the death *in vitro* of numerous tumor cell types, including liver cancer, cholangiocarcinoma and multiple myeloma (44,51-53). Furthermore, parthenolide has been demonstrated to enhance the sensitivity of cancer cells to therapy, including liver cancer cell sensitivity to cisplatin (54,55). The present study revealed that H₂O₂ increased Bax expression and decreased Bcl-2 expression. Parthenolide significantly prevented these H₂O₂-mediated alterations in osteoblasts. A direct interaction may exist between parthenolide and H₂O₂ in the culture medium, and parthenolide may inhibit H₂O₂-induced apoptosis in osteoblasts.

Nrf2 is a transcription factor that regulates the intracellular metabolism of endogenous and exogenous substances in the

oxidative stress response. Nrf2 binds to the antioxidant response element (1) and up-regulates ARE-associated antioxidative genes, including endogenous antioxidants, phase II detoxification enzymes and transcripts encoding intracellular genes that determine cell survival or death (56). The sustained expression of Nrf2 has important functions in restoring cellular homeostasis (57). A number of natural or synthetic small molecule compounds have been confirmed to activate the Nrf2/ARE signaling pathway, resulting in protection of cells from toxic or carcinogenic material-induced cell damage (58). Nrf2/ARE pathway activation results in the expression of cytoprotective enzymes, including NQO1 and HO-1 (58). Therefore, Nrf2 has an essential role in protection against oxidative stress. The results of the present study indicated that parthenolide increased Nrf2, HO-1 and NQO1 expression in H₂O₂-induced osteoblasts. Therefore, parthenolide may suppress osteoblast apoptosis by reducing oxidative stress.

In conclusion, the results of the present study confirmed that parthenolide increased viability and inhibited apoptosis in H₂O₂-induced osteoblasts. The decrease in apoptosis mediated by parthenolide may be via a reduction in oxidative stress.

Additionally, parthenolide was demonstrated to increase the expression of the osteogenesis-associated genes Runx2, OPN, OCN and Col-1 in H₂O₂-induced osteoblasts. Therefore, parthenolide may have potential as a therapeutic drug for osteoporosis. Future studies are required to further validate the key findings of the present study. The effects of parthenolide may be verified *in vivo* in osteogenic tissues and a rat model under oxidative stress, and additional cell lines may be used to validate the important conclusions of the present study.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors' contributions

WM wrote the main manuscript. WM and ZZ performed the experiments. WM and ZZ designed the study. ZZ performed data analysis. WM and ZZ contributed to manuscript revisions and all authors reviewed the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the fifth People's Hospital of Yuhang District.

Consent for publication

Informed consent was approved by both the 8-year-old patient and his guardian.

Competing interests

The authors declare that they have no competing interests.

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