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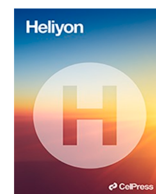

Research article

Study on the mechanism of naringin in promoting bone differentiation: In *vitro* and *in vivo* study

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ABSTRACT

Objective: Osteoporosis is a common clinical bone disease that occurs most frequently in middle-aged and elderly people. Various traditional herbal medicine formulations have shown significant benefits in models of osteoporosis. In this study, we aim to investigate the osteogenic efficacy of naringin (NRG) in the osteoporotic state.
Design: We treated Bone marrow stromal cells (BMSCs) with various concentrations of NRG for 3 and 7 days. BMSC proliferation was measured by the MTT assay. The effect of NRG on the osteogenic differentiation of BMSCs was detected by ALP and alizarin red staining. The effect of NRG on the BMP2/Runx2/Osterix signaling pathway was analyzed by using real-time PCR. The effect of NRG on the oestrogen receptor was measured by Enzyme-linked immunosorbent assay. *In vivo* animal experiments were performed by micro-computed tomography and ALP immunohistochemistry to determine the ectopic osteogenic effect of NRG sustained-release nanoparticles in a mouse model of osteoporosis.
Results: NRG promoted the proliferation and osteogenic differentiation of BMSCs. Moreover, it also activated the BMP2/Runx2/Osterix signaling pathway. When NRG sustained-release nanoparticles were added *in vivo* in animal experiments, we found that NRG sustained-release nanoparticles had better ectopic osteogenic effects in a mouse model of osteoporosis.
Conclusions: NRG induced osteoblastic differentiation of BMSCs by activating the BMP2/Runx2/Osterix signaling pathway and promoted the regulation of oestrogen receptor pathway protein expression, and NRG sustained-release nanoparticles exerted a more significant *in vivo* ectopic osteogenic effect in an osteoporosis mouse model. Therefore, naringin is expected to be developed as a novel treatment for inducing osteogenesis, because of its ubiquitous, cost-efficient, and biologically active characteristics. However, further research is needed on how to improve the pharmacokinetic properties of naringin and its specific mechanism.

1. Introduction

In the 1990s, osteoporosis (OP) was defined as a group of metabolic bone diseases associated with increased bone fragility and an

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increased risk of fracture, due to microstructural degradation and low-mineral density of bone tissue [1]. According to recent statistics [2–4], many people suffer from osteoporosis, and adverse consequences such as fractures not only reduce the quality of life of these patients but also cause a heavy economic burden for society and families. Previous studies [5,6] reported a close correlation between alveolar bone loss and bone mineral density (BMD). Cross-sectional studies conducted in South Korea [7,8] indicated a significant correlation between osteoporosis and decreased alveolar bone density, with similar associations in China [9], Japan [10], Jordan [11], Greece [12] and other countries. In view of this, osteoporosis is considered as a disease that may affect periodontal disease, residual alveolar ridge resorption, and implant success and has therefore drawn great interest from periodontists. Therefore, research and development of a drug that can effectively treat osteoporosis may resolve the symptoms of low alveolar bone density, periodontitis and poor postimplant prognosis in the dental field.

At present, in the clinical treatment of osteoporosis, the available drugs include drugs inhibiting bone resorption and drugs promoting bone formation, but side effects limit their use; therefore, natural drug research has become a hot spot. In the context of orthopaedics, screening of common Chinese herbal medicines has identified agents that inhibit bone resorption and promote bone regeneration; this is one way to find new drugs to treat osteoporosis [13,14]. It is worth noting that the composition of traditional Chinese medicines is complex, and the efficacy of such components involves various mechanisms of action. However, OP, a multifactor bone metabolic disease, cannot be treated overnight but requires adjustment over time and treatment of both the symptoms and root causes.

Naringin (NRG) is a polymethoxyflavonoid extracted from citrus plants with anti-inflammatory, antioxidation, anticancer and osteogenesis-stimulating effects. Estabell [15] reported that naringin inhibited RANKL-induced nuclear factor kappa β (NF-κB) activation and ERK phosphorylation, thus inhibiting osteoclast formation and bone resorption, confirming the protective effect of naringin on bone loss. There is additional evidence [16] that the naringenin increases BMP-2 expression and enhances osteogenic responses through phosphoinositide 3-kinase (PI3K), AKT, c-Fos/c-Jun, and AP-1-dependent signaling pathways. Considering the important effects of naringin on multiple bone reconstruction-related pathways, including antioxidant free radicals, mitogen-activated protein kinase (MAPK), Wnt/β-catenin, and bone morphogenic protein 2/SMAD (BMP2/SMAD), it may be a promising drug for the future treatment of bone-related diseases [17].

Based on this, we report here that the naringin monomer promotes the osteogenic differentiation of Bone marrow stromal cells (BMSCs) via BMP2/Runx2/Osterix signaling pathway and the estrogen receptor pathway, and further reveal that naringin induced osteogenesis more effectively in ectopic experiments with naringin sustained-release granules in the gastrocnemius muscle pocket in a mouse model of osteoporosis.

2. Materials & methods

2.1. Cell culture and proliferation

Mouse primary BMSCs were isolated and purified from bone marrow cells of the osteoporotic mouse model. After being cultured, digested, and passaged, P3 BMSCs with good growth status were selected and inoculated in α-MEM complete medium (1 % double antibody, 0.4 % FBS, HyClone, USA) and routinely cultured for 24 h. Naringin at different concentrations (1 and 10 μmol/L) (batch number: 110722–200809, China Institute for Drug and Biological Products Control) was added, and the control group (CNT group), 1 μmol/L group and 10 μmol/L group were incubated for 24 h, 48 h and 72 h. At each time point, added 20 μl MTT solution [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl bromide) (5 g/L; Amresco, USA) to continue the routine culture for 4 h, discard the supernatant, added dimethyl sulfoxide (DMSO, S. Sigma-Aldrich, USA) to dissolve, and measured the absorption value of each well at 570 nm.

2.2. Osteogenesis induction

P3-generation BMSCs with a good growth rate were selected for 24 h after inoculation, and the medium was replaced with osteogenic induction medium containing 1 or 10 μmol/L naringin or control medium used for osteogenic induction (1 % each antibody, 10 % FBS, 50 mg/L ascorbic acid, 10 mmol/L dexamethasone, 10 mmol/L β-sodium glycerophosphate). The cells were grown in an environment with 5 % CO_2 and a temperature of 37 °C.

2.3. Osteogenic differentiation followed by histology

2.3.1. ALP activity was measured and quantified

The cell culture plates were removed on days 3 and 7 after induction of osteogenesis in the presence of naringin, and the old medium was aspirated and gently rinsed with PBS buffer as previously described. The cells were fixed in 4 % paraformaldehyde for 15 min at room temperature, stained according to the instructions of the ALP staining kit (Cell Biolabs, USA), rinsed in PBS and photographed under a microscope. The protein standard and BCA working solution were configured as indicated by the kit, and the OD value was measured at 450 nm in triplicate using a microplate reader.

2.3.2. Alizarin red staining

Cell culture plates were removed on days 3 and 7 after induction of osteogenesis in the presence of naringin, and the old medium was aspirated and gently rinsed with PBS buffer as previously described. Then, 4 % paraformaldehyde was used for fixation for 15 min at room temperature, and the cells were stained with 2 % alizarin red dye solution (Sigma–Aldrich, USA), decolorized with sodium

phosphate solution for 30 min, photographed and observed under a microscope. Finally, the OD value was determined at 595 nm using a microplate reader, and the experiment was performed in triplicate.

2.4. Molecular biology

2.4.1. Real-time quantitative PCR (RT-PCR)

The osteogenesis-related genes bone morphogenetic protein 2 (BMP-2), Runt-related transcription factor 2 (Runx2), osteopontin (OPN) and Osterix in BMSCs were selected and tested by RT-PCR with β-actin as the internal reference. Gene primer sequences are shown in Table 1. On the 3rd and 7th day of osteogenic induction, samples were collected after cell lysis in enzyme-free EP tubes, and total RNA was extracted using TRIzol reagent (Invitrogen, USA). The ratio of A260 nm/A280 nm and RNA content were determined with a nucleic acid protein analyser, and the RNA was reverse transcribed according to the instructions of the Prime Script™ RT reagent Kit with gDNA Eraser (Takara, USA) with the $2^{-\Delta\Delta CT}$ method used to analyze the results.

2.4.2. Enzyme-linked immunosorbent assay (ELISA)

P3 generation BMSCs with a logarithmic growth period and good growth status were selected. After inoculation and culture, the culture medium was discarded, the cells were rinsed twice in PBS, and the culture medium was replaced to establish the control, ICI 182.780 (an oestrogen pathway blocker 10 μM), naringin and naringin + ICI 182.780 groups (10 μM). After 24 h, the cells were digested by trypsin (Thermo Scientific, USA), centrifuged at 4 °C and 2000 r/min, left in PBS, gently rinsed, added to an appropriate amount of RIPA lysis buffer, and lysed on ice for 30 min. After centrifugation at 4 °C and 12000 r/min, the supernatant was removed and kept at −80 °C. The expression level of each histone was determined according to the β-catenin, ERα, and ERβ enzyme-linked immunization kits (Sigma–Aldrich, USA) according to the manufacturer's instructions.

2.5. Preparation and performance analysis of slow-release particles

2.5.1. Preparation and Morphological observation

The drug Poly L Lactic Acid (PLLA) was acquired from China Hanggai Biotechnology Co., Ltd. Chitosan (chitosan, CS, DAC z85 %, 200 cps) was purchased from Shandong AK Biotechnology Co., Ltd. According to the dual emulsification method [18], composite synthesis of naringin/PLLA (Poly L Lactic Acid, Hanggai Biological Corp., China) particles and NRG/PLLA/CS (chitosan: DAC Z 85 %, 200 cps; Shandong AK Biotech Co., Ltd., China) sustained-release particles was performed.

2.5.2. In vitro release experiments

Phosphate-buffered saline (PBS, 0.1 mM, pH = 7.4, containing 0.5 % T80) was used to simulate blood, and the two different types of particles were dissolved in 5 mL of deionized water by the dialysis bag technique [19]. In *vitro* release studies were performed within 24 h. The dialysis device was placed perpendicular to the constant temperature oscillation incubator, and 0.5 mL of the release medium was removed at the specified time and replaced with the same volume, temperature, and pH of release medium to ensure that the total volume was unchanged. All measurements were repeated in triplicate.

2.6. In vivo implantation experiment in mice

2.6.1. Experimental grouping and methods

All animals were subjected to humane care, and all methods were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethics Committee of Jinan University (batch No. 20210720-09). Adult wild-type (WT) C57BL 6/J female mice (8–10 weeks old) were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China) and bred in a nonspecific pathogen (SPF)-grade animal house at the School of Basic Medicine of Jinan University. An osteoporosis mouse model was prepared by ovary removal in 16 female mice, and bone modelling was verified by X-ray double-energy rays. After intraperitoneal injection of the mice with 1 % sodium pentobarbital (3.5 mg/100 g) for anaesthesia, NRG/PLLA was transplanted into the left gastrocnemius pocket in that NRG group, and NRG/PLLA/CS was transplanted into the right gastrocnemius pocket in the NRG nanoparticle group; the soft tissue was then sutured.

2.6.2. Gross observation and immunohistochemical staining of ALP

Mice were individually killed by air embolization at 2 weeks after surgery, the gastrocnemius pockets were removed, and the size of

Table 1
Primers used in the qRT-PCR experiments.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
BMP-2	CATGCCATTGTTCAGACG	TGTACTAGCGACACCCACA
OPN	GAAGTTTCGCAGACCTGACAT	GTATGCACCATTCAACTCCTCG
Runx2	GCACCCAGCCCATAATAGA	TTGGAGCAAGGAGAACCC
Osterix	GCCTACTTACCCGTCTGACTTT	GCCCACTATTGCCAACTGC
GAPDH	CATCTCTTGCTCGAAGTCCA	ATCATGTTTGAGACCTTCAACA

bone-calcified tissue at the graft site was visually observed. The removed bone-calcified tissue was fixed in 4 % formaldehyde, followed by immunohistochemical staining with ALP and visualization under an inverted microscope.

2.6.3. Micro-CT 3D reconstruction and detection of bone parameter

A Bruker Skyscan 1172 micro-CT scanner (Micro Photonics Inc, USA) was used, and 3D reconstruction of calcified bone tissue, comparison of two-dimensional cross-sections, and detection of bone mineral density (BMD), bone volume fraction (BVF), and trabecular bone thickness (Tb. Th) were then performed.

2.7. Statistical analysis

All data obtained from the experiments are expressed as mean ± standard deviation (SD). Comparisons between the two groups were analyzed using Student's t-test, and comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). Statistical analysis was performed using SPSS 22.0. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Naringin promotes the proliferation and osteogenic differentiation of BMSCs

Using MTT assays, we examined the effect of 0, 1, and 10 μmol/L naringin on BMSC proliferation. From 24 h to 72 h, naringin significantly promoted BMSC proliferation, and the effect was enhanced over time ($P < 0.05$) (Fig. 1 A).

To test the effect of naringin on the osteogenic differentiation of BMSCs, we performed ALP staining and alizarin red staining. Positive ALP staining is visualized as blue–purple staining under the microscope. We investigated the effect of naringin on BMSC differentiation by staining for mineralization. On days 3 and 7 of osteogenic differentiation, more calcium deposits accumulated in the cells of the 10 μmol/L naringin group, and the mineralized deposits in each group increased over time. ALP quantification further revealed the strongest ALP activity in the 10 μmol/L group, which had values significantly higher than those of the control and 1 μmol/

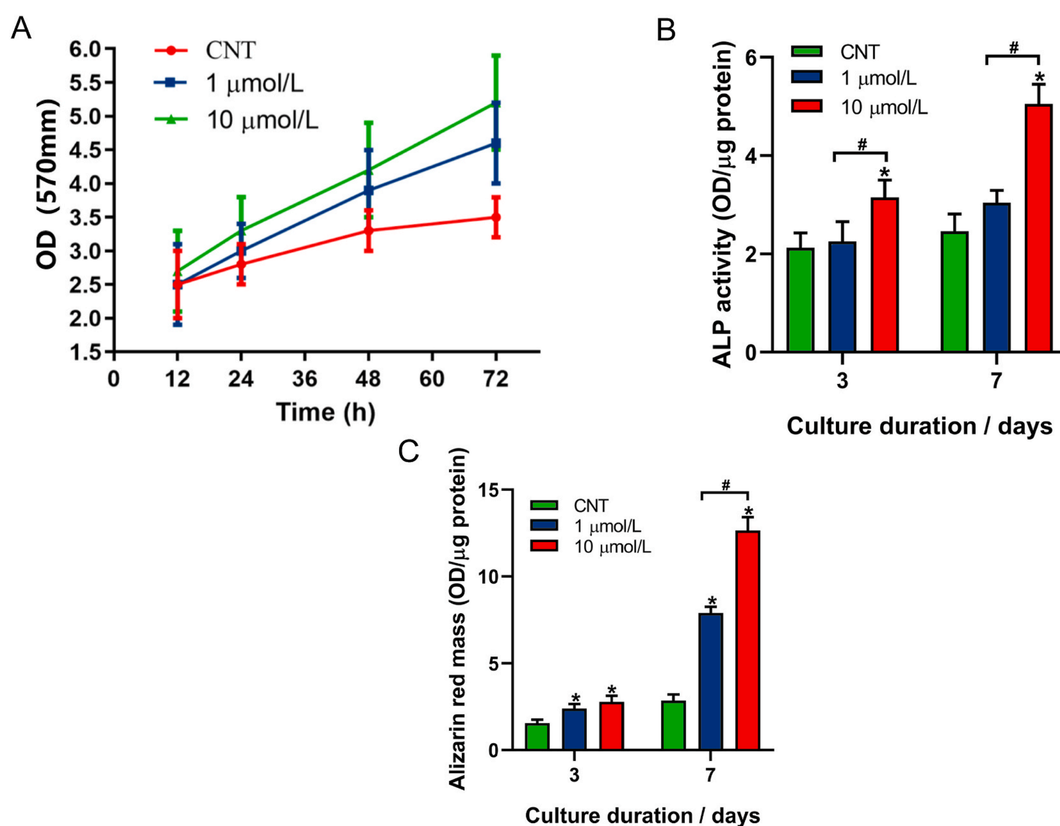

Fig. 1. Naringin promotes the proliferation and osteogenic differentiation of BMSCs. (A) Results of MTT assays, Effects of 0, 1, and 10 μmol/L naringin on the proliferation of BMSCs, $P < 0.05$. (B) Results of ALP analysis. * Compared with the CNT group, $P < 0.05$; # compared with the 1 μmol/L group, $P < 0.05$. (C) Results of alizarin red analysis. * Compared with the CNT group, $P < 0.05$; # compared with the 1 μmol/L group, $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

L groups at different time points (P < 0. 05) (Fig. 1 B). This result showed that naringin can increase the ALP activity of BMSCs in a dose-dependent manner, and the 10 μmol/L naringin can better improve ALP activity and promote BMSCs osteogenesis.

Positive alizarin red staining was observed as scarlet staining under the microscope. We measured the mineralization of BMSCs in the presence of naringenin by measuring the number of calcified nodules and evaluating the positively stained area. On the 7th day of osteogenic differentiation, cells in both the 1 μmol/L and 10 μmol/L groups showed significant positive expression of alzarin red. Further alizarin red quantification showed that the 1 and 10 μmol/L groups had significantly higher values on days 1 and 7 than the control (P < 0. 05), and the mineralization in the 10 μmol/L group was also significantly higher than that in the 1 μmol/L group at 7 d (P < 0.05) (Fig. 1C). This result also proved that naringin can induce osteoblastic differentiation of BMSCs, which was the same as the conclusion of ALP staining.

3.2. Naringin activates the BMP2/Runx2/Osterix signaling pathway

To elucidate the mechanism of naringin in the osteogenic differentiation of BMSCs, we evaluated the effect of naringin on the expression of factors associated with the BMP2/Runx2/Osterix signaling pathway. Real-time PCR revealed that 1 μmol/L and 10 μmol/L naringin significantly increased mRNA expression of BMP-2, Runx2 and Osterix genes (P < 0.05), whereas differential expression of OPN gene mRNA on day 7 was observed (P < 0.05). In the real-time PCR experiment, all indicators except Osterix showed differential expression in the 10 μmol/L group compared with the 1 μmol/L group (P < 0.05) (Fig. 2A–D). Our results demonstrated that NRG may

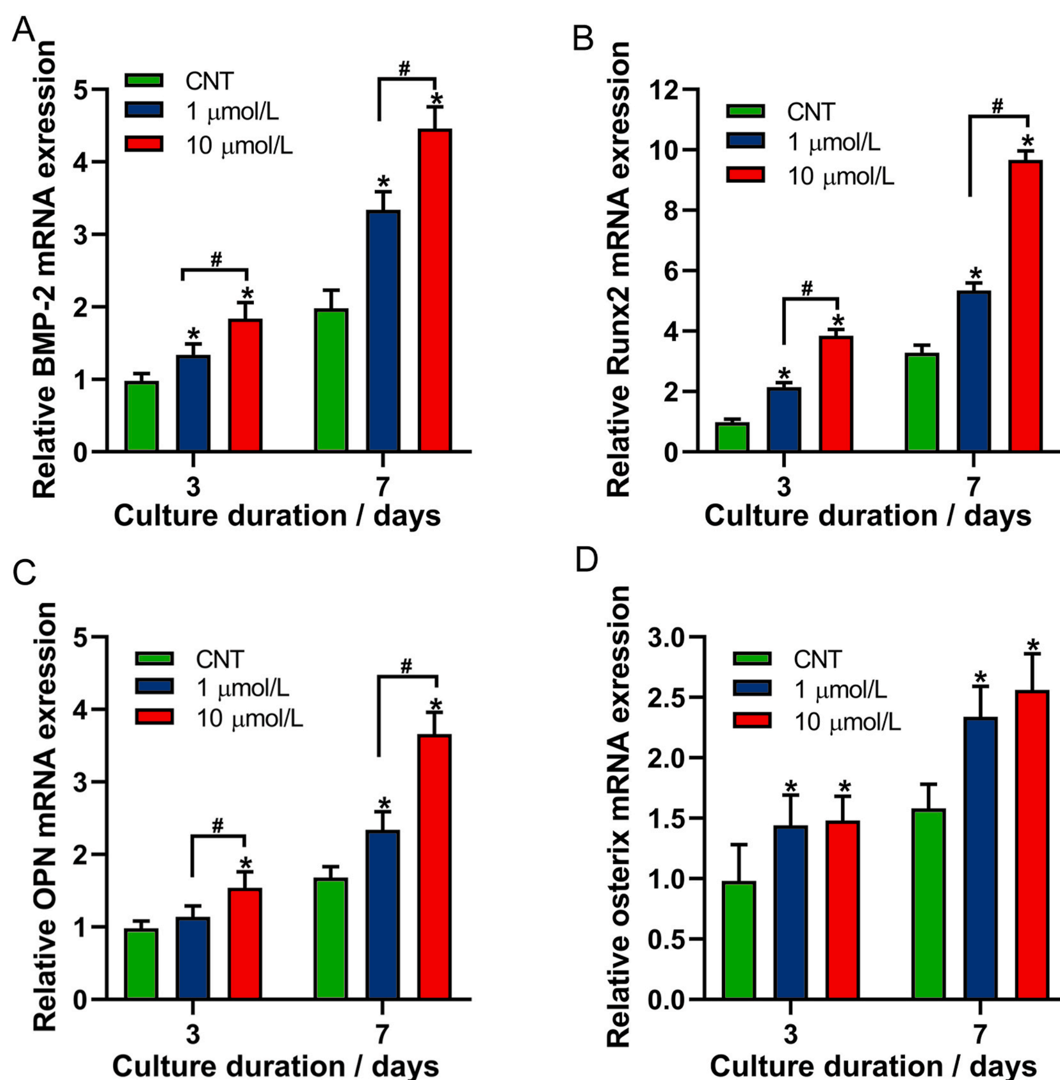

Fig. 2. Naringin activates the BMP2/Runx2/Osterix signalling pathway. BMSCs were cultured in 0, 1, and 10 μmol/L naringin for 3 and 7 days, respectinely, and Real-time PCR detection of osteogenesis-related genes (including BMP-2(A), Runx2(B), OPN(C), and Osterix mRNA(D)) expression levels.

induce osteoblastic differentiation of BMSCs by activating the BMP2/Runx2/Osterix signaling pathway.

3.3. Naringin activates the oestrogen receptor pathway

In the following experiment, to determine if the action of naringin is mediated by the ER, we further observed the effect of ICI182.780 (an oestrogen pathway blocker) on the oestrogen receptor protein expression of BMSCs induced by naringin to assess the contribution of the drug to the process. ELISA showed increased expression of both oestrogen receptor proteins in BMSCs under naringin intervention ($P < 0.05$), and estrogen receptor (ER)β expression changed more significantly (Fig. 3 B). Interestingly, ICI182.780 inhibited naringenin, and the naringin + ICI182.780 combination group showed a decrease in ERα and ERβ protein expression compared with that in the simple drug intervention group ($P < 0.01$). The decrease in ERβ expression was particularly obvious, as the expression levels were lower than those in the control group ($P < 0.05$) (Fig. 3A–B).

3.4. NRG sustained-release granules exerted a more significant in vivo ectopic osteogenic effect

Finally, NRG/PLLA particles and NRG/PLLA/CS sustained-release particles were separately implanted into the gastrocnemius muscle pouch of mice to investigate whether naringin could induce bone formation *in vivo*. Before administration, a in *vitro* release assay was performed. The findings indicated that NRG/PLLA group exhibited a sudden release at the beginning of 4 days and then a gradually slowing release of naringin, with a cumulative release of 85 %, while the nearly linear release behavior of NRG/PLLA/CS group proved that the NRG sustained-release granules could sustain a constant release of naringin in 3 weeks (Fig. 4).

After 2 weeks, mouse gastrocnemius pouches were dissected to compare the newly formed bone calcified tissue. Under the macroscopic observation, the calcified tissue size was larger in the NRG nanoparticle group (Fig. 5A–B). Immunohistochemical staining of ALP showed that the effect of NRG nanoparticles on bone was significantly enhanced, and the positive expression areas were mostly distributed in newly formed bone tissue and newly formed connective tissue, while the NRG group showed less ALP expression, mostly distributed in the surrounding connective tissue (Fig. 5C–D). A micro-CT scan of the removed bone-calcified tissue was performed, and the cross-section comparison showed that the trabecular bone was sparse and had low bone density in the NAR group compared to the NRG nanoparticle group. Morphological data for the trabecular bone in the NRG nanoparticle group showed the BMD, BVF, and Tb.Th values were significantly higher than those in the NRG group ($P < 0.01$) (Fig. 6A–E). Taken together, these results all suggested that compared to the naringin, naringin sustained-release granules had better ectopic osteogenic effects in a mouse model of osteoporosis.

4. Discussion

The three main biological mechanisms that regulate bone tissue adaptation to the external environment are bone growth, bone moulding, and bone reconstruction. Bone reconstruction is the only regulatory mechanism that plays a strong role in adulthood. It involves osteoclast (OC)-mediated bone formation and osteoblast (OB)-mediated bone resorption, which work together to complete the processes of bone tissue repair, bone homeostasis maintenance, mechanical stimulation and adaptation. As the global population ages, osteoporotic fractures gradually become a major health and societal problem [20]. Geriatric and postmenopausal osteoporosis are extremely common in the population, which has important implications for the clinical success of intraosseous dental implants. The long-term clinical effectiveness of these implants is closely related to the direct contact of the implant and the bone, and osseointegration is needed to achieve primary stability of the implant after implantation.

The contribution of MSCs to bone formation and bone integration has been demonstrated previously [21–23]. During the functional differentiation of BMSCs, bone morphogenetic protein (BMP), a member of the transforming growth factor β (TGF-β) superfamily, is considered the central inducer of bone induction [24]. The TGF-β/BMP signaling pathway is of great importance in mammalian skeletal development [25]. In related reports, BMP-Smad signalling promoted almost every step of osteoblast differentiation and

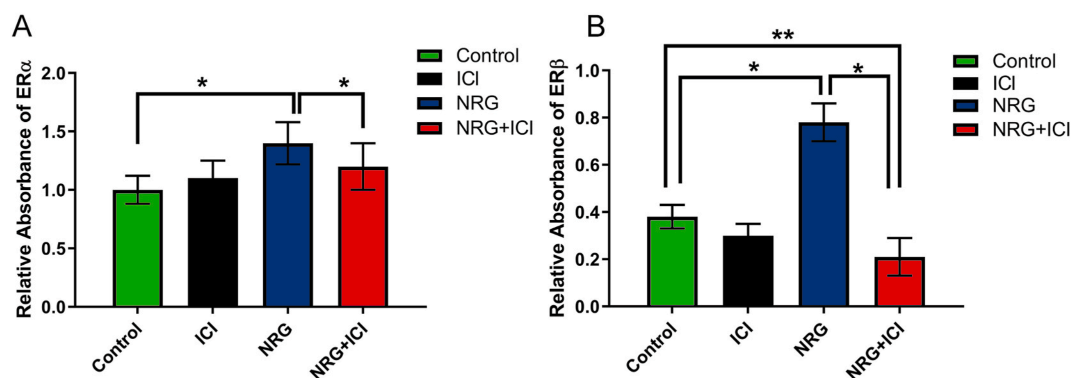

Fig. 3. Naringin activates the oestrogen receptor pathway. Effects of naringin on the expression levels of ERα (A) and ERβ (B).

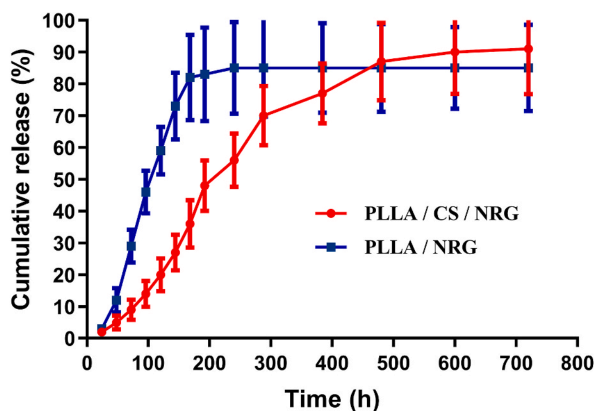

Fig. 4. In *vitro* release curve of naringin sustained-release nanoparticles.

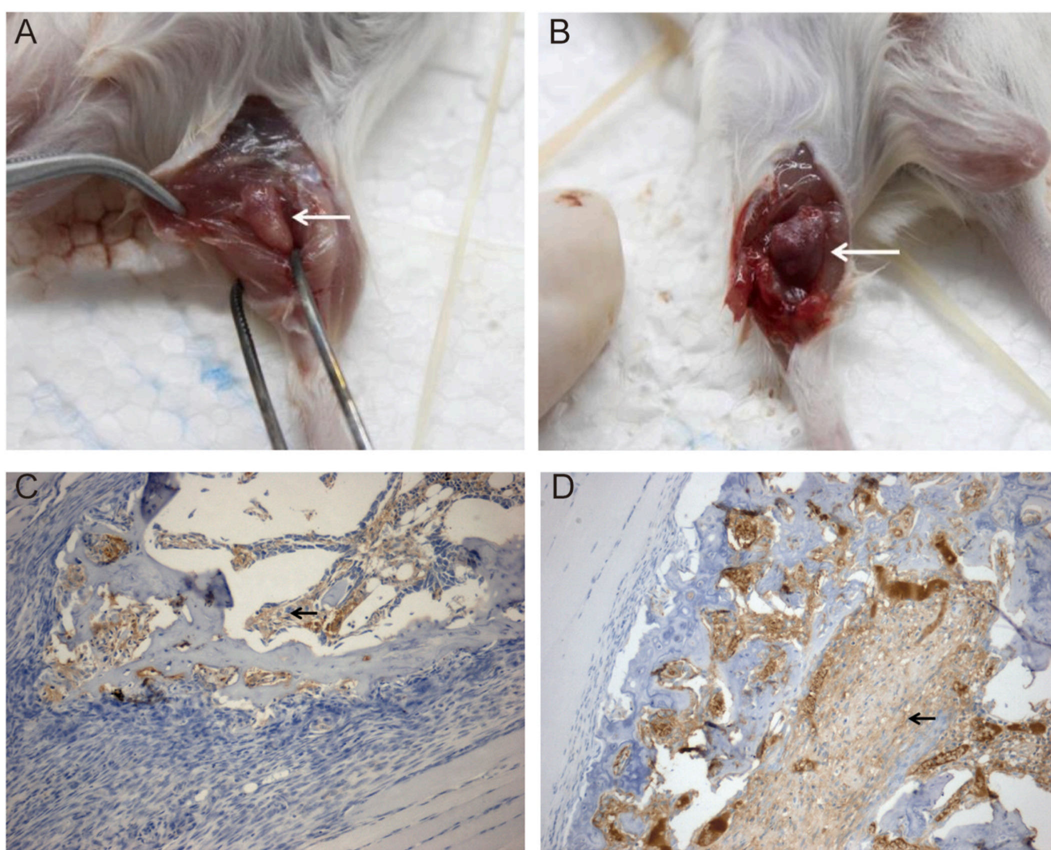

Fig. 5. NRG sustained-release granules exerted a more significant *in vivo* ectopic osteogenic effect in an osteoporosis mouse model. Bone calcified tissue was removed surgically after NRG (A) and NRG nanoparticle (B) treatment. Immunohistochemical staining of ALP after NRG (C) and NRG nanoparticle (D) treatment in osteoporosis mouse models at 2 weeks (scale bar = 400 μm).

maturation [26]· The osteogenic differentiation of multipotent mesenchymal progenitors depends on the sequential expression of two key transcription factors, namely, transcription factor 2 (Runx2) and Osterix, which are important target molecules in the BMP-Smad signaling pathway; their role in bone metabolism was reported by Long [27], Chatakun [28] and Yan [29]. Researchers have also confirmed that the activation of the BMP2/Runx2/Osterix signaling pathway is essential for osteogenic differentiation and bone defect repair. Therefore, the components involved in BMP activation are good targets for the development of anti-osteoporosis drugs. This study confirmed that the BMP2 gene was significantly expressed after the application of naringin, with concurrent mRNA expression of a series of signalling factors in the BMP2/Runx2/Osterix pathway, including BMP2, Runx2, Osterix, OPN, etc. The conclusion that

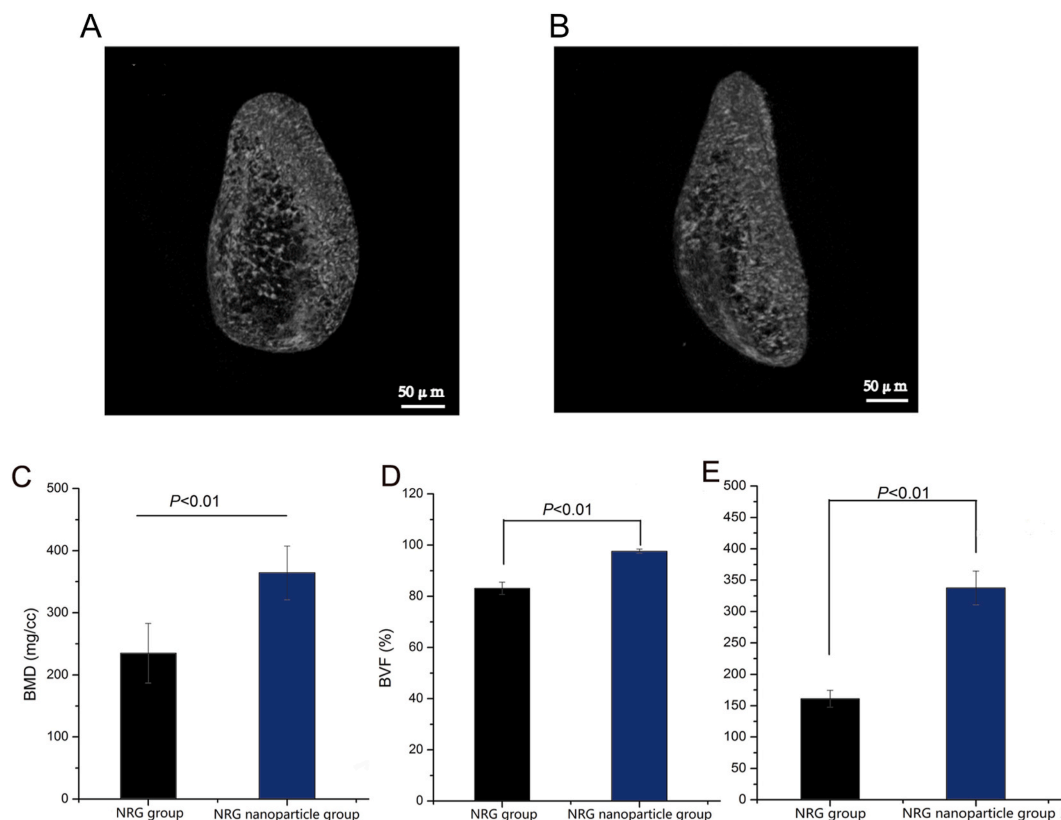

Fig. 6. Micro-CT 3D reconstruction and bone parameter detection: A shows the 3D reconstruction for NRG nanoparticles, B shows the 3D reconstruction for NRG (scale bar = 1 mm), C shows the BMD, D shows the BVF, and E shows the Tb.Th. Statistical analysis of the bone parameters.

naringin promotes bone formation through the activation of the BMP2/Runx2/Osterix signaling pathway compensates for the gap in current studies on the signaling pathways that promote osteogenesis.

Bone loss related to oestrogen mostly occurs in trabecular bone (also known as cancellous bone) and cortical bone. It is clear that sex steroid deficiency, especially when oestrogen levels drop, directly mediates OC and OB formation, but the former increase is due to coupling during bone remodelling; in fact, bone formation is still lower than bone resorption [30,31]. This finding indicates that ERα expression is high in cortical bone, whereas ERβ is strongly expressed in cancellous bone. In bone, ERα mainly mediates the effects of oestrogen and is therefore thought to be more important for skeletal homeostasis [32,33]. Nonetheless, Er β, as a regulator of the transcriptional activity of ERα, antagonizes many ERα-driven effects, and its role in maintaining bone homeostasis cannot be ignored [34]. This has mostly been confirmed in a murine model [30]. In this study, ER expression was upregulated under naringin intervention, and ER expression also decreased when the expression of the oestrogen pathway was inhibited. Considering these results, we speculate that naringenin indeed has an effect on the oestrogen receptor pathway during bone metabolism and has a more pronounced effect on Erβ, one of the receptors in this pathway. In a clinical report by Wu et al. [35], Er β was shown to bind to an oestrogen response element located at site 488 of the AT sequence-binding protein 2 (SATB2) gene, which upregulates SATB2 expression, further regulating BMSC differentiation and inhibiting senescence through the ER β-SATB2 pathway. This view may explain the observations related to Erβ in this study.

To date, most osteoporosis treatments have focused on approaches to block bone resorption, but studies have shown that promoting bone formation or dual-effect therapy can achieve better efficacy in improving the bone microstructure and reducing fracture risk than antiresorptive therapy [36,37]. Multicomponent, multitarget, and low-toxicity traditional Chinese herbs have been widely used in the clinical treatment of bone fractures in China and have been shown to have many advantages [38]. Among these agents, naringin is a polymethoxylated flavonoid found in a variety of fruits, vegetables, barks, tea trees, stems, leaves and other natural products, with extensive natural sources. As reported previously [39–41], naringin has anti-inflammatory, antioxidation, metabolic regulation, and antidiabetic efficacy and has very high medicinal value. A meta-analysis [42] showed that naringin improved BMD in an osteoporosis rat model better than oestrogen. Naringin relies on bone marrow MSCs [43,44] and human periodontal membrane stem cells [45]. In addition, naringin has oestrogen-like activity, which highlights its osteogenic value. Previously, studies on potential signaling pathways by which naringin promotes BMSC proliferation and osteogenic differentiation are mostly focused on Wnt/β-catenin, the RANKL/OPG system signaling pathway, and the PI3K/AKT/mTOR signaling pathway [43,46]. Wnt and β-catenin are important regulatory factors that promote the expression of BMP-2, which is a protein that can promote osteoblast differentiation. Naringin can

stimulate the Wnt/β-catenin pathway, thereby upregulating BMP-2 and promoting the formation of osteoblasts. Besides, the formation of osteoclasts is mediated by RANK/RANKL and bone protein (OPG). Naringin has been shown to inhibit osteoclast differentiation and bone resorption by inhibiting the RANK/RANKL pathway [43]. What is more, naringin also can enhance the differentiation of osteoblasts by activating the PI3K/AKT/mTOR pathway, which may be related to its ability to induce autophagy in osteoblasts [46]. Consistent with previous studies, we found in this study that naringin enhances BMSC osteogenesis in *vitro*. More importantly, we further confirmed the effects of naringin on osteogenesis-related factor expression in the BMP2/Runx2/Osterix signaling pathway.

At present, the resolution of medical CT still cannot meet the imaging requirements for accurate research in animal experiments, but the resolution of micro-CT is on the order of microns. This approach gradually becomes the "gold standard" for evaluating bone morphology and bone microstructure in animal experiments [47]. Micro-CT not only provides clear imaging but also enables scanning and imaging without killing animals or damaging specimens. Notably, micro-CT can satisfy the need to avoid arbitrary angle tomographic images, and from this perspective, it overcomes the difficulty of limited manipulation or observation due to specimen shape/structure in pathological sections [48]. In the context of osteoporosis, the Tb.Th value was decreased, and BMD was decreased. In 3D reconstructions and bone parameter detection using micro-CT, the NAR sustained-release nanoparticle group showed higher BMD, BVF, and Tb.Th values. These data indicate that the osteogenic effect of NAR in the sustained-release form is more satisfactory.

Although researchers have reported excellent prospects for naringin in the treatment of osteoporosis, its limitations of poor water solubility and low oral bioavailability must be addressed before implementing a treatment. This study confirmed that the preparation of naringin in sustained-release particles can effectively improve its pharmacokinetic properties to optimize the delivery and lasting effects of naringin. Thus, naringin, a natural compound derived from Chinese herbal medicines, indeed has therapeutic potential and clinical application value for osteoporosis-related diseases.

Data availability statement

Data will be made available on request.

Ethics statement

This study was reviewed and approved by the Animal Experimental Ethics Committee of Jinan University, with the approval number: 20210720-09.

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CRediT authorship contribution statement

Xian Li: Writing – original draft, Investigation. **Xiaojun Zhou:** Writing – original draft, Formal analysis. **Zhanyu Huang:** Writing – original draft, Formal analysis. **Kexiao Chen:** Validation. **Xinrong Jiang:** Data curation, Visualization. **Renfa Lai:** Writing – review & editing. **Zejian Li:** Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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