Blackcurrant extract promotes differentiation of MC3T3-E1 pre-osteoblasts

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Received March 6, 2024; Accepted May 23, 2024

DOI: 10.3892/br.2024.1810

Abstract. Osteoporosis risk increases in menopausal individuals owing to the decrease in estrogen secretion. Blackcurrant extract (BCE) ameliorates osteoporosis; however, the underlying mechanisms are unclear. Furthermore, although BCE has phytoestrogenic activity, its effects on osteoblasts are unknown. In the present study, we investigated BCE-mediated attenuation of osteoporosis using mouse MC3T3-E1 pre-osteoblasts, with a focus on osteogenesis. After treating MC3T3-E1 cells with BCE for 48 h, cell proliferation was assessed using Cell Counting Kit-8. Levels of osteoblast differentiation markers, namely alkaline phosphatase activity and total collagen content in the cells, were evaluated after 3 and 14 days of BCE treatment, respectively. The expression of genes encoding osteoblast differentiation markers, including collagen type I (*Col-I*), alkaline phosphatase (*Alp*), bone γ-carboxyglutamate protein (*Bglap*), and runt-related transcription factor 2 (*Runx2*), was evaluated using reverse transcription-quantitative polymerase chain reaction. Mineralization of the cells was evaluated using Alizarin Red staining. Femoral tissues of ovariectomized (OVX) rats with or without 3% BCE were stained using ALP to evaluate osteogenic differentiation in femoral tissue. After treating MC3T3-E1 cells with BCE, cell proliferation had increased. BCE treatment increased Alp activity and total collagen content. Moreover, the expression of *Col-I*, *Alp*, *Bglap*, and *Runx2* increased in BCE-treated cells. Furthermore, when MC3T3-E1 cells were treated with BCE for 21 days, the levels of calcified nodules increased. Alp staining intensity was stronger in the epiphyses on femoral tissue of OVX rats treated with 3% BCE than in those of untreated OVX rats. The results suggest that BCE may promote osteogenesis by inducing osteoblast differentiation.

Introduction

Blackcurrant (*Ribes nigrum L.*) contains polyphenols, particularly four anthocyanins: Cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside and delphinidin-3-rutinoside. Additionally, it contains large amounts of vitamins A, C and E, as well as small amounts of each of the B vitamins. Moreover, it contains abundant minerals, such as calcium, iron, magnesium, phosphorus, potassium and zinc (1). These compounds elicit beneficial health effects, including increased blood flow, cancer suppression and prevention of glaucoma, eye strain and lifestyle-related diseases, such as obesity and diabetes mellitus (2,3).

Phytoestrogens are plant-derived substances that exhibit effects similar to those of endogenous estrogens. Multiple phytoestrogens, including isoflavones and resveratrol, have been identified (4,5). Decreased estrogen secretion associated with increased age and menopause increases risk of developing disorders, including decreased blood vessel function, dyslipidemia and osteoporosis (6-8). Our previous studies showed that blackcurrant extract (BCE) and its anthocyanins exert phytoestrogenic activity via signaling through estrogen receptors α and β (9,10) and alleviate some menopausal symptoms, including arteriosclerosis, hair loss, skin aging and dyslipidemia (11-14).

Bone is remodeled by constantly being resorbed and formed by osteoblasts. Notably, imbalance between bone resorption and formation causes a decrease in bone density, leading to osteoporosis (15). Moreover, bone remodeling is a complex process involving several hormones, including estrogen (16,17). Osteoporosis refers to a condition in which bone mass decreases and bone structure deteriorates, weakening bone strength and increasing susceptibility to fractures (18,19). Considering that estrogen regulates bone metabolism, osteoporosis is more likely to occur following menopause-associated decrease in estrogen, a condition called postmenopausal osteoporosis (20).

During early stage osteoporosis, patients present almost no symptoms, being difficult to diagnose this condition.

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Abbreviations: Actb, β-actin; Alp, alkaline phosphatase; BCE, blackcurrant extract; Bglap, bone γ-carboxyglutamate protein; Col-I, collagen type I; E2, 17β-estradiol; OD, optical density; OVX, ovariectomized; Runx2, runt-related transcription factor 2

Key words: blackcurrant, osteoblast, osteoporosis, phytoestrogen

Thus, it is important to consume foods rich in calcium, such as fish, dairy products and seaweed during menopause. The intake of phytoestrogens, such as equol, which is produced by metabolism of soy isoflavones contained in soybeans and soybean foods by intestinal bacteria, is effective in preventing postmenopausal osteoporosis (21,22). Blackcurrant has also been shown to reduce the risk of osteoporosis and improve trabecular bone mass in young mice (23,24). Additionally, blackcurrant alleviates osteoporosis in humans, and clinical trials are currently ongoing to validate its efficacy (25).

Estrogen acts on both osteoclasts and osteoblasts; its deficiency in menopause is hypothesized to accelerate bone resorption by osteoclasts and decrease bone mass (26). Although blackcurrant has been shown to inhibit osteoclastogenesis, studies on its effects on osteogenesis are lacking (27). Additionally, the mechanism by which blackcurrant alleviates osteoporosis remains unclear. As osteoblasts are sensitive to estrogen, the present study aimed to investigate the phytoestrogenic effects of BCE on osteoblast proliferation and differentiation using mouse pre-osteoblastic MC3T3-E1 cells. Notably, these cells produce large amounts of collagen, differentiate into osteoblasts and ultimately form bone (28-30).

In osteoblasts, expression of differentiation markers, such as collagen type I (*Col-I*), alkaline phosphatase (*Alp*), bone γ-carboxyglutamate protein (*Bglap*) and runt-related transcription factor 2 (*Runx2*), increase depending on the extent of differentiation (31). Osteoblasts in the late stage of differentiation produce mineralized deposits (calcified nodules) that can be stained with Alizarin Red (32,33). To the best of our knowledge, the present study is the first to assess the health effects of BCE on osteoblast differentiation.

Materials and methods

Reagents and cell culture. BCE powdered extract was CaNZac-35 (Koyo Mercantile Co., Ltd.), containing a high concentration of polyphenols and anthocyanins (37.6 and 38.0% w/w, respectively) (10). 17β-estradiol (E2) was purchased from Sigma-Aldrich (Merck KGaA). The mouse pre-osteoblast cell line MC3T3-E1 was obtained from the Health Science Research Resources Bank (Osaka, Japan). MC3T3-E1 cells were maintained in α-MEM (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% (v/v) FBS (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 μg/ml streptomycin (FUJIFILM Wako Pure Chemical Corporation). Cell culture experiments were conducted at 37˚C in a humidified incubator under 5% CO_2.

Cell treatment. MC3T3-E1 cells ($1x10^4$ cells/well) were seeded in six replicates in 96-well plates and cultured overnight in α-MEM supplemented with 10% (v/v) FBS. The medium was replaced with phenol red-free α-MEM supplemented with 5% (v/v) charcoal-stripped FBS (Thermo Fisher Scientific, Inc.). Cells were cultured for 48 h at 37˚C in the presence or absence of 0.2, 1.0, 5.0 or 20.0 μg/ml BCE or 10 nM E2. E2 was used as a positive control to examine the phytoestrogenic effect of BCE on MC3T3-E1 cells (32). Treatment duration and the E2 concentration of 10 nM were selected based on previous studies (29,33). The morphology of MC3T3-E1 cells was analyzed under a phase-contrast microscope (CK40; Olympus Corporation; magnification, x100) with an Anyty™ digital microscope camera (3R-DKMCO4; Three R Solution Corp. Japan).

Proliferation assay. Quantification of cell proliferation was performed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) according to the manufacturer's instructions. CCK-8 solution was added to the wells, and incubated for 30 min at 37˚C. Absorbance was measured at 450 nm using a Benchmark microplate reader (Bio-Rad Laboratories, Inc.).

Alp assay. MC3T3-E1 cells ($5x10^4$ cells/well) were seeded in duplicate in 24-well plates and cultured overnight as aforementioned. The growth medium was replaced with phenol red-free α-MEM, supplemented with 5% (v/v) charcoal-stripped FBS. The cells were cultured for 72 h with BCE or 10 nM E2 as aforementioned, based on previous studies (30,34). After washing with 0.1 M Tris-HCl buffer (pH, 9.8), 100 μl 0.1% (v/v) Triton X-100 in 0.1 M Tris-HCl buffer (pH, 9.8) was added to the medium and the plates were stored at -80˚C. The plates were rapidly thawed at 37˚C and assayed using the LabAssay™ ALP kit (FUJIFILM Wako Pure Chemical Corporation). Protein concentrations were determined using a Takara BCA Protein Assay kit (Takara Bio, Inc.). Absorbance relative to Alp activity and protein concentrations were measured at wavelengths of 405 and 570 nm, respectively, using a Benchmark microplate reader (Bio-Rad Laboratories, Inc.).

Quantification of total collagen. MC3T3-E1 cells ($1x10^5$ cells/well) were seeded in duplicate in 12-well plates and cultured overnight in α-MEM supplemented with 10% (v/v) FBS. The growth medium was replaced with differentiation medium [phenol red-free α-MEM supplemented with 5% (v/v) charcoal-stripped FBS and Osteoblast-Inducer Reagent (Takara Bio, Inc.)], according to the manufacturer's protocol. Cells were cultured in the presence or absence of BCE or 10 nM E2, as aforementioned, and the medium was replaced every 3 days. Collagen staining was performed on day 14 using a Sirius Red/Fast Green Collagen Staining kit (Iwai Chemicals Co. Ltd.), according to the manufacturer's instructions. Cells were washed with PBS, followed by the addition of 0.5 ml Kahle fixative at 22˚C for 10 min. Dye solution was added to culture plates and incubated at 22˚C for 30 min. Cells were rinsed with 0.5 ml distilled water until the solution was colorless. Digital images were acquired using a phase-contrast microscope (CK40; magnification x40) with an Anyty™ digital microscope camera. Following microscopy, the dye was eluted. The optical density (OD) of the eluted dye solution was measured at 540 and 605 nm using a spectrophotometer (U-5100; Hitachi High-Technologies Corporation). The calculation formulas followed the kit manufacturer's instructions: Collagen (μg/section)=OD_{540}-(OD_{605} x 0.291)/0.0378; non-collagen protein (μg/section)=OD_{605}/0.00204.

Reverse transcription-quantitative PCR (RT-qPCR). MC3T3-E1 ($2x10^5$ cells/well) were seeded in 6-well plates and cultured overnight as aforementioned until 80% confluent. The medium was replaced with phenol red- and serum-free α-MEM with or without BCE or 10 nM E2, as aforementioned. After incubating at 37˚C for 24 h, cells were washed

twice with PBS. Total RNA was extracted using the RNeasy Mini kit (Qiagen GmbH), according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara Bio, Inc.), according to the manufacturer's protocol. *Col-I*, *Alp*, *Bglap* and *Runx2* mRNA expression levels were quantified via RT-qPCR using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara Bio, Inc.). Thermocycling conditions were as follows: 30 sec at 95˚C, followed by 40 cycles of 5 sec at 95˚C and 30 sec at 60˚C. Transcription levels were normalized to β-actin (*Actb*). Primers (5'→3') were as follows: *Col-I* forward, GAGCGG AGTACTGGATCG and reverse, GCTTCTTTTCCTTGG GGTT (31); *Alp* forward, GATCATTCCCACGTTTTCACA TT and reverse, TTCACCGTCCACCACCTTGT (31); *Bglap* forward, GCGCTCTGTCTCTCTGACCT and reverse, AAG CAGGGTCAAGCTCACAT (31); *Runx2* forward, AGCGGC AGAATGGATGAGTC and reverse, ACCAGACAACACCTT TGACG (35) and *Actb* forward, CATCCGTAAAGACCT CTATGCCAAC and reverse, ATGGAGCCACCGATCCAC A (36). PCR specificity was determined using melting curve analysis. All samples were analyzed in duplicate. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (37).

Mineralization assessment. Calcified nodule formation was assessed using Calcification Evaluation set (PG Research), according to the manufacturer's instructions. Briefly, $1x10^5$ cells/well were seeded in duplicate in 12-well plates containing differentiation medium (Osteoblast-Inducer Reagent). Cells were treated as aforementioned with BCE or 10 nM E2 on day 21 and the medium was replaced every 3 days. At the end of treatment, medium was removed and wells were washed with PBS. Cells were fixed with neutral buffered 10% formalin at 22˚C for 10 min, followed by washing with distilled water. The cells were stained with 1.0 ml Alizarin Red solution at 22˚C for 30 min. Micrographs were acquired using a fluorescence microscope (FSX100; Olympus Corporation; magnification, x40). After removing the water, 0.5 ml calcified nodule lysate [5% (v/v) formic acid] was added and the plates were stirred for 10 min at 22˚C to elute the dye. The absorbance of the eluate was measured at 450 nm using a spectrophotometer (U-5100).

Animals and treatments. Ovariectomized (OVX) and sham-operated female Sprague-Dawley rats (OVX, n=6; sham-operated rats, n=6; age; weight, ~240 g; 12 weeks; CLEA Japan, Inc.) were housed in plastic cages in air-conditioned rooms (23˚C; 50% humidity) under a 12/12-h light/dark cycle at the Institute for Animal Experiments of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). All experimental procedures were approved by the Animal Research Committee of Hirosaki University (approval no. G18003). Our previous study showed that 3% BCE has phytoestrogenic effects in rats (10). All rats were fed AIN-93M diet, with or without 3% BCE (Oriental Yeast Co., Ltd.) and were divided into three groups (n=3/group): Sham, OVX and OVX + 3% BCE. All rats had free access to food and water. After 3 months, the animals were euthanized by anesthesia with isoflurane (induction, 5%; maintenance, 3%), followed by decapitation, then femurs were excised and fixed in 10% (v/v) formaldehyde at 22˚C for 1 week, demineralized in 10% (v/v) EDTA-2Na (pH, 7.2) at 4˚C for 3 weeks and embedded in paraffin. Femur sections (4 μm) were routinely passed through xylene and descending ethanol series before Alp staining.

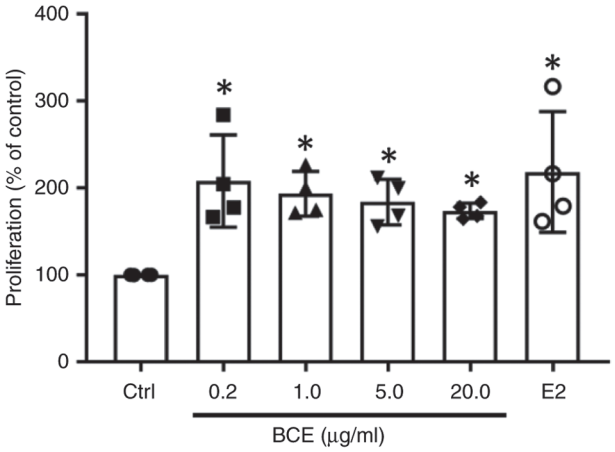

Figure 1. BCE or 10 nM E2 significantly increases MC3T3-E1 cell proliferation. *P<0.05 vs. Ctrl. BCE, blackcurrant extract; E2, 17β-estradiol; Ctrl, control.

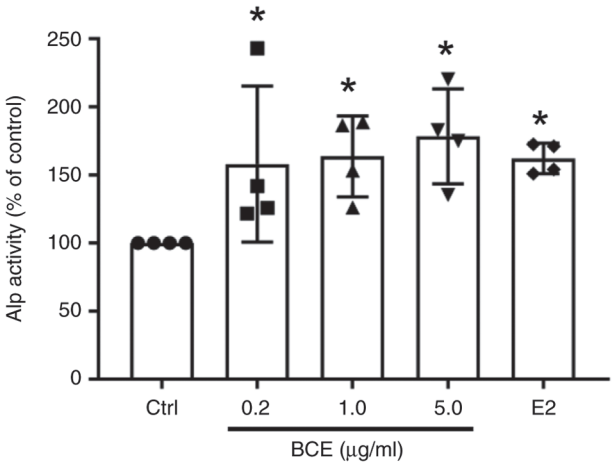

Figure 2. BCE and E2 treatment increases Alp activity in MC3T3-E1 cells. *P<0.05 vs. ctrl. BCE, blackcurrant extract; E2, 17β-estradiol; ctrl, control; Alp, alkaline phosphatase.

Alp staining. To measure femoral Alp activity, femurs were stained with a TRAP/ALP kit (FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer's instructions. Nuclei were stained with hematoxylin at 22˚C for 5 min. Specimens were examined and photographed using a fluorescence microscope (FSX100; Olympus Corporation; magnification, x40).

Statistical analysis. All data are expressed as mean ± SD from four independent experiments. Graphs were generated using GraphPad Prism (version 7.03; Dotmatics). Significant differences were determined using Kruskal-Wallis analysis and Steel post hoc test via Bell Curve in Excel software (version 3.2; Social Survey Research Information Co., Ltd.). P<0.05 was considered to indicate a statistically significant difference.

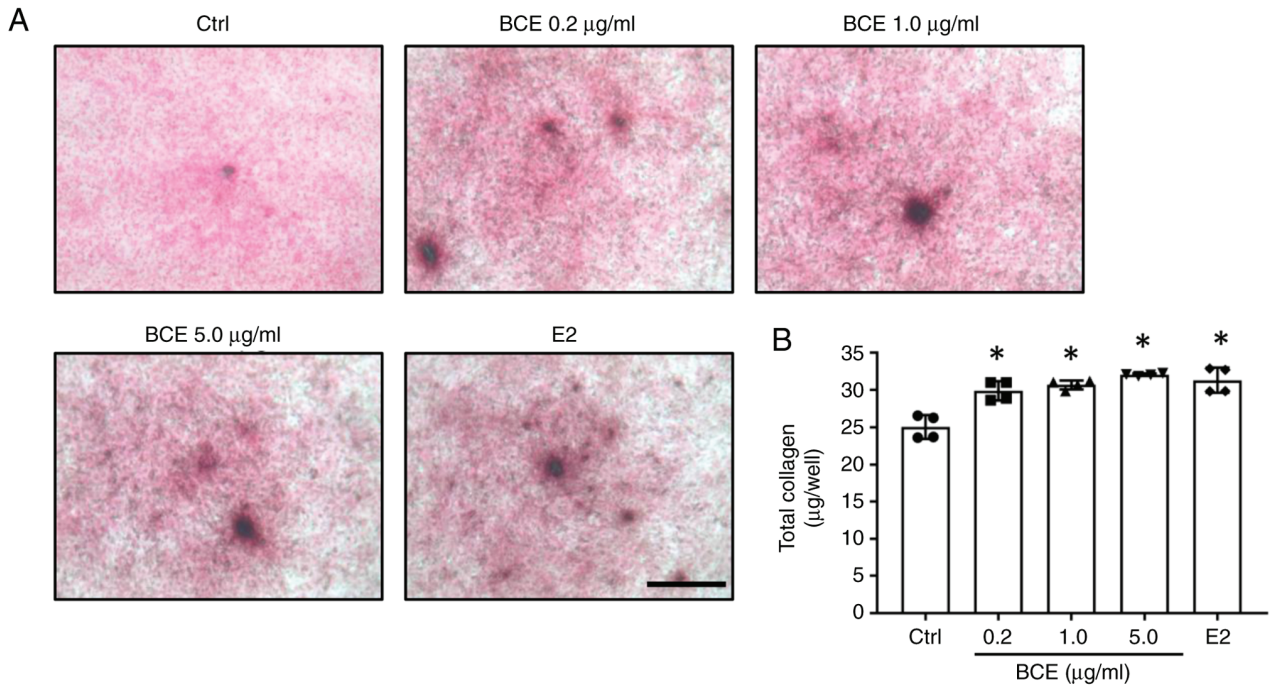

Figure 3. Effect of BCE on collagen production in MC3T3-E1 cells. (A) MC3T3-E1 cells were treated with BCE or 10 nM E2 for 14 days. The cells were stained using a Sirius Red/Fast Green Collagen Staining kit. Collagen was stained red. Scale bar, 500 μm. (B) Pigment was extracted and measured via spectrophotometry. *P<0.05 vs. ctrl. BCE, blackcurrant extract; E2, 17β-estradiol; ctrl, control.

Results

BCE promotes MC3T3-E1 cell proliferation. The present study investigated the effect of BCE on MC3T3-E1 cell proliferation. Cells were treated with 0.2, 1.0, 5.0 or 20.0 μg/ml BCE, which showed phytoestrogenic effects in our previous studies (12,13) or 10 nM E2 as a positive control. Cell proliferation increased significantly (P<0.05) after treatment with all concentrations of BCE or 10 nM E2 (Fig. 1). However, there was no notable difference in cell morphology following treatment with BCE or E2 (Fig. S1).

BCE enhances Alp activity in MC3T3-E1 cells. MC3T3-E1 cells were treated with 0.2, 1.0 or 5.0 μg/ml BCE or 10 nM E2 and cultured for 72 h. Alp activity increased significantly (P<0.05) in a dose-dependent manner following treatment with BCE and increased significantly (P<0.05) upon treatment with E2 (Fig. 2).

BCE enhances total collagen production in MC3T3-E1 cells. MC3T3-E1 cells were treated with 0.2, 1.0 or 5.0 μg/ml BCE or 10 nM E2 and cultured for 14 days. Total collagen was quantified using Sirius Red staining. Total collagen increased significantly (P<0.05) after treatment with all concentrations of BCE and 10 nM E2 (Fig. 3).

BCE upregulates expression of osteogenic markers in MC3T3-E1 cells. MC3T3-E1 cells were incubated with 0.2, 1.0 or 5.0 μg/ml BCE or 10 nM E2 for 24 h. Bone differentiation marker expression was measured using RT-qPCR. *Col-I*, *Alp*, *Bglap* and *Runx2* expression increased significantly (P<0.05) following treatment with all concentrations of BCE and 10 nM E2 (Fig. 4).

BCE promotes mineralization in MC3T3-E1 cells. MC3T3-E1 cells were treated with 0.2, 1.0 or 5.0 μg/ml BCE or 10 nM E2 and cultured for 21 days. Cells were stained with Alizarin Red to assess the extent of mineralization, which revealed the presence of calcified nodules (Fig. 5A). Levels of calcified nodules increased significantly (P<0.05) following treatment with all concentrations of BCE and 10 nM E2 (Fig. 5B).

BCE induces osteoblast differentiation in vivo. We investigated whether BCE induces osteoblast differentiation in the femoral tissue of OVX rats, which were used as a menopausal model. As Alp activity increases in the region surrounding the epiphysis, where active osteoblast differentiation occurs, rat femurs were stained to assess Alp activity. Compared with that in the Sham group, Alp staining intensity was weak in the epiphyses of rats in the OVX group. However, Alp staining intensity was stronger in the epiphyses of OVX rats treated with 3% BCE than in those of untreated OVX rats (Fig. S2, arrows).

Discussion

BCE inhibits osteoclast differentiation (27); however, its effects on osteoblasts are unknown. Therefore, the present study focused on the effect of BCE on differentiation of MC3T3-E1 pre-osteoblasts into osteoblasts.

Administration of phytoestrogens or 10 nM E2 significantly enhances MC3T3-E1 cell proliferation (28,38). Here, BCE or E2 treatment increased the proliferation of MC3T3-E1 cells, which was consistent with previous findings (28,38). Overall, the increase in MC3T3-E1 cell proliferation may be due to the phytoestrogen activity of BCE.

Estrogen activity has been shown to increase Alp activity and collagen secretion in MC3T3-E1 cells (31). In

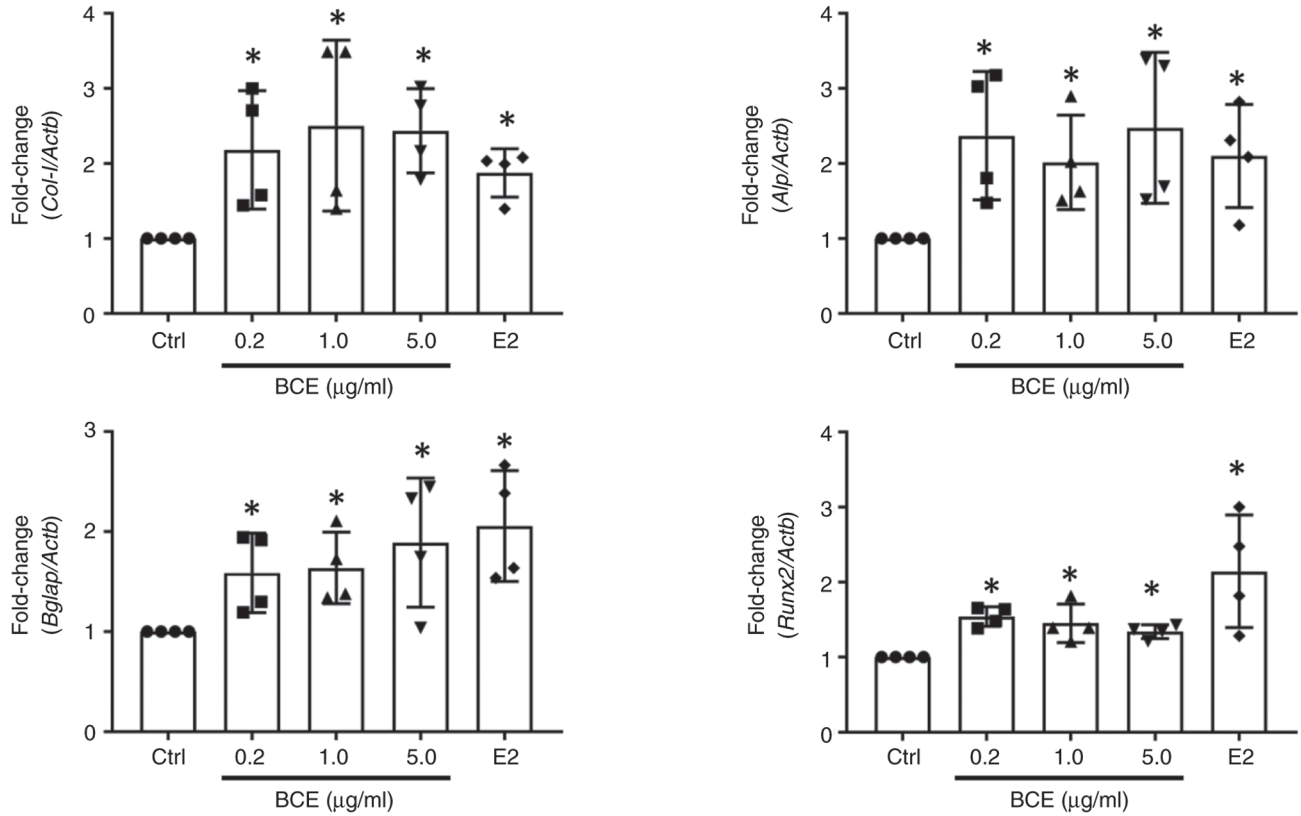

Figure 4. BCE and E2 increases expression of osteogenesis marker genes. MC3T3-E1 cells were treated with BCE or 10 nM E2 for 24 h. *P<0.05 vs. ctrl. BCE, blackcurrant extract; E2, 17β-estradiol; ctrl, control; Col-I, collagen type I; Alp, alkaline phosphatase; Bglap, bone γ-carboxyglutamate protein; Runx2, runt-related transcription factor 2; Actb, β-actin.

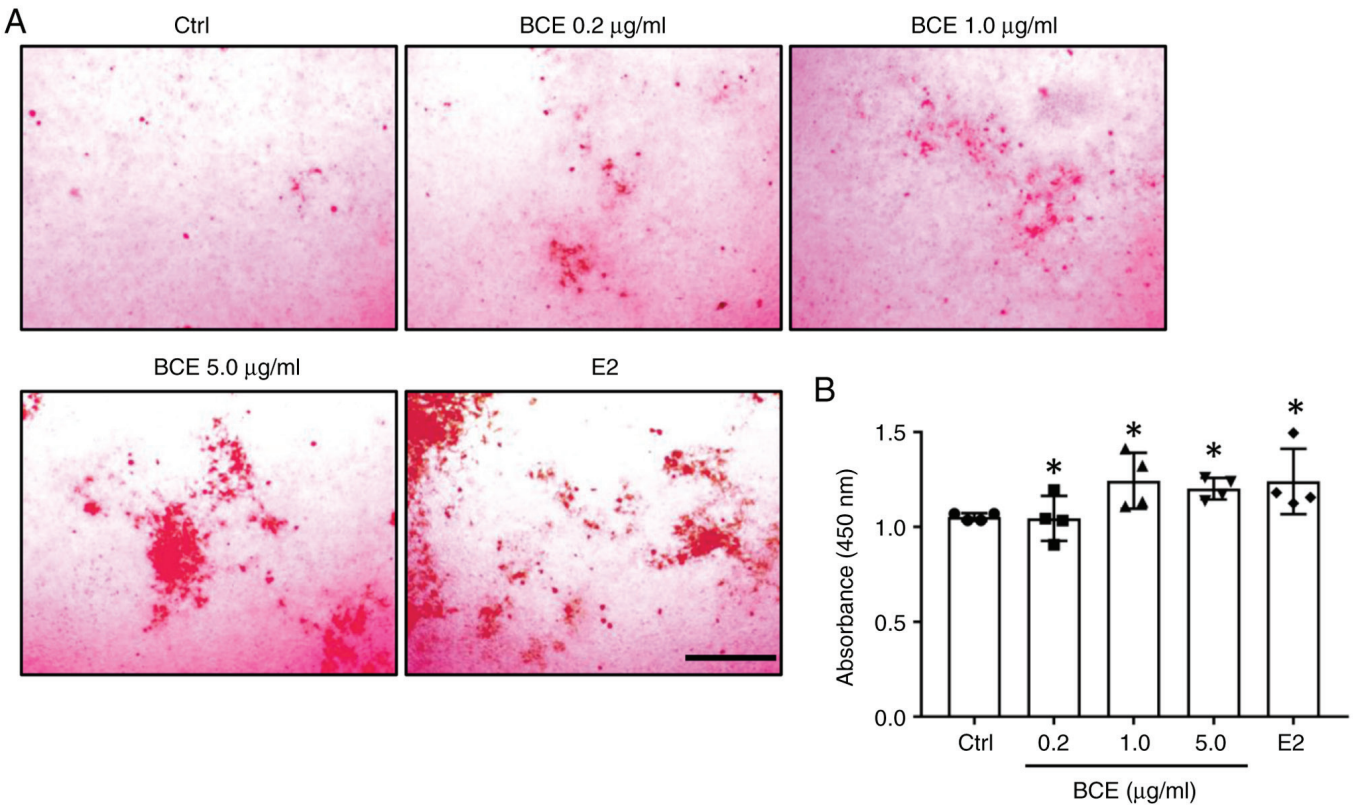

Figure 5. Effect of BCE treatment on the mineralization of MC3T3-E1 cells. (A) MC3T3-E1 cells were treated with BCE or 10 nM E2 for 21 days. The cells were stained with mineralization evaluation set. Mineralization areas were stained red. Scale bar, 500 μm. (B) Pigment was extracted with calcified nodule lysate and measured using a spectrophotometer. *P<0.05 vs. ctrl. BCE, blackcurrant extract; E2, 17β-estradiol; ctrl, control.

the present study, BCE increased Alp activity and collagen secretion in MC3T3-E1 cells in a concentration-dependent manner. *Col-I*, *Alp*, *Bglap* and *Runx2* are known osteoblast differentiation markers (31). Here, BCE and E2 increased the expression of all four genes, which may be due to phytoestrogen activity.

Consistent with the present findings, several studies have shown that anthocyanin induces osteoblast differentiation, with increased expression of *Col-I*, *Alp*, *Bglap* and *Runx2* (33,39,40). *Runx2* is a transcription factor required for osteoblast differentiation and is expressed at an early stage of differentiation (41). By contrast, *Col-I*, *Alp* and *Bglap* are the earliest biomarkers of mature osteoblast differentiation (42,43). In addition to increased expression of these genes, there was an increase in Alp activity and collagen protein secretion in the present study. Furthermore, there was an increase in the levels of calcified nodules. Collectively, these results suggested that BCE induced osteogenesis and differentiation of pre-osteoblasts into osteoblasts.

Our previous study revealed the half-maximal inhibitory concentration for estrogen receptor α is ~10 nM for E2 and ~5 μg/ml for BCE; relative binding affinity of BCE is 0.06% relative to E2 (10). Therefore, BCE may have significantly lower estrogenic activity in MC3T3-E1 cell than 10 nM E2.

Although BCE has been shown to be effective in animal models of osteoporosis (23,24,27), to the best of our knowledge, there has been no study on the effect of BCE on osteoblast differentiation *in vivo*. As the present *in vivo* experiment was simple, more detailed studies using micro-computed tomography, are required. Additionally, it is key to isolate osteoblasts from BCE-treated and untreated OVX rats to examine the effect of BCE on osteoblast differentiation. Moriwaki *et al* (27) reported that bilberry, BCE and their anthocyanins prevent osteoporosis by inhibiting excessive osteoclastogenesis. Although osteoblasts were also used in the aforementioned study, BCE treatment caused no change in cell differentiation. Research findings indicate that estrogen and phytoestrogen enhance osteoblast differentiation (30,31,34); however, results may differ depending on the estrogen sensitivity of osteoblasts. In the present study, phytoestrogen effects were observed in estrogen-sensitive MC3T3-E1 cells. Experiments using estrogen receptor inhibitors were not performed in the present study as our previous studies demonstrated that BCE has phytoestrogenic effects (9,10). However, it is unclear which molecules of BCE exhibit phytoestrogenic effects on MC3T3-E1 cells. Therefore, detailed component analysis of BCE and experiments using estrogen receptor inhibitors against its candidate molecules are necessary in future. Additionally, it is important to verify the effect of different compounds in BCE on osteoclasts. Polyphenols include anthocyanin-rich plants and attenuate osteoporosis (24,27). Notably, their mechanism is reported to include the activation of signaling via the Wnt/β-catenin, transforming growth factor-β/bone morphogenetic protein 2, mitogen-activated protein kinase and PI3K/AKT pathways (44,45). The present study focused on phytoestrogen activity. However, the intracellular signaling mechanism is complex, as phytoestrogens also activate PI3K/AKT, Src/ERK1/2, and nuclear factor κB via the estrogen receptor (46,47). Moreover, BCE likely interacts with these factors via estrogen receptors (10). A microarray analysis of breast cancer MCF7 cells with high expression of estrogen receptors showed that BCE activates multiple pathways (10).

Menopause is associated with increased risk of osteoporosis owing to decreased estrogen secretion. Although hormone replacement therapy is available for treatment of osteoporosis, it is associated with complications, including increased risk of breast cancer (48,49). Research findings indicate that the intake of phytoestrogens, such as soy isoflavones and equol, prevents and ameliorates osteoporosis (21,22). However, as phytoestrogens are not suitable for people who are allergic to soybeans, the anti-osteoporotic effect of blackcurrant is key. Pre-clinical and clinical trials are required to validate the present findings in humans.

In conclusion, BCE and E2 promoted cell proliferation, increased Alp activity and collagen production, upregulated expression of osteoblast differentiation markers, and enhanced mineralization and differentiation of pre-osteoblasts into osteoblasts. Overall, although BCE has phytoestrogenic effects, the present data suggest that it also affected osteoblast differentiation. Collectively, the results suggested that BCE may ameliorate osteoporosis during menopause.

Acknowledgments

Not applicable.

Funding

The present study was partially supported by the Japan Society for the Promotion of Science KAKENHI (grant no. 20K02402).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

NN, IO and KH designed the study, performed the experiments and analyzed the data. NN wrote the manuscript. IO edited the manuscript. All authors have read and approved the final manuscript. NN and KH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Animal Research Committee of Hiroaki University (Hirosaki, Japan; approval no. G18003).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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