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Research Article

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Blackcurrant extract promotes the differentiation of MC3T3-E1 pre-osteoblasts

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Abstract

Background The risk of developing osteoporosis in menopausal women increases as estrogen secretion decreases. Blackcurrant extract (BCE) has been reported to ameliorate osteoporosis; however, the mechanisms underlying this phenomenon are unclear. Furthermore, although BCE has phytoestrogenic activity, its effects on osteoblasts are unknown. In this study, mouse pre-osteoblast MC3T3-E1 cells were used to investigate BCE-mediated attenuation of osteoporosis, with a focus on osteogenesis.

Methods and Results After treating MC3T3-E1 cells with BCE for 48 h, the total number of cells was counted, and no conspicuous differences were observed. The levels of osteoblast differentiation markers, alkaline phosphatase activity, and total collagen content increased in BCE-treated cells on days 3–14. Additionally, the expression of genes encoding osteoblast differentiation markers, including collagen type I, alkaline phosphatase, osteocalcin, and runt-related transcription factor 2, increased in BCE-treated cells. At 21 days of treatment, calcified nodule levels were increased.

Conclusions These findings show that BCE may promote osteogenesis by inducing osteoblast differentiation.

Keywords
Blackcurrant; Osteoblasts; Osteoporosis; Phytoestrogen.

Abbreviations
Actb, actin beta; Alp, alkaline phosphatase; BCE, blackcurrant extract; Bglap, bone gamma-carboxyglutamate protein; Col-I, collagen type I; E2, 17β-estradiol; OD, optical density; OVX, ovariectomized; Runx2, runt-related transcription factor 2; SD, standard deviation.
**Introduction**

Blackcurrant (*Ribes nigrum* L.) contains abundant polyphenols, particularly the four anthocyanins cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, and delphinidin-3-rutinoside. In addition, it contains large amounts of vitamins A, C, and E, as well as small amounts of each of the B vitamins. Moreover, it has 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, eyestrain, and lifestyle-related diseases such as obesity and diabetes [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]. Women are known to be at an increased risk of developing disorders including decreased blood vessel function, dyslipidemia, and osteoporosis, as estrogen secretion decreases owing to aging and menopause [6-8]. These menopausal symptoms are often treated with estrogen replacement therapy; however, a complementary dietary approach is necessary to reduce side effects. Our previous studies reported that blackcurrant extract (BCE) exerted phytoestrogenic activity via signaling through both the estrogen receptors α and β [9, 10] and that it alleviated some menopausal symptoms, including arteriosclerosis, hair loss, skin aging, and dyslipidemia [11-14].

Bones are remodeled by being resorbed and formed by osteoblasts constantly. It is believed that when the balance is lost between bone resorption and bone formation, bone density decreases, leading to osteoporosis [15]. Moreover, bone remodeling is very complex, involving multiple hormones such as 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]. As estrogen regulates bone metabolism, osteoporosis is more likely to occur
when estrogen levels decrease during menopause in a process called postmenopausal osteoporosis [20]. Fragility fractures associated with osteoporosis are a major health problem worldwide. More than 40 million Americans over the age of 50 are at risk of such fractures, and with changing demographics, it has been estimated that this number will at least double by 2040 [21]. This is also associated with significant mortality; 25% of people over the age of 50 who have an osteoporotic hip fracture die within a year [21].

Since osteoporosis is difficult to recognize, eating foods rich in calcium, such as small fish, dairy products, and seaweed, is important during menopause. The intake of phytoestrogens such as equol, which is produced by the metabolism of soy isoflavones contained in soybeans and soybean foods by intestinal bacteria, is reportedly effective in preventing postmenopausal osteoporosis [22, 23]. Blackcurrant also has been shown to reduce osteoporosis and improve trabecular bone mass in young mice [24, 25]. It has been reported to alleviate osteoporosis in humans, and clinical trials are underway [26, 27]; thus, its efficacy is an ongoing research topic.

Estrogen acts on both osteoclasts and osteoblasts; its deficiency in menopause is thought to accelerate bone resorption by osteoclasts and decrease bone mass. While blackcurrant has been shown to inhibit osteoclastogenesis, studies on its effects on osteogenesis are lacking [28].

The mechanism by which blackcurrant alleviates osteoporosis remains unclear. As osteoblasts are sensitive to estrogen, mouse pre-osteoblastic MC3T3-E1 cells were used in this study to investigate the phytoestrogenic effects of BCE on osteoblast proliferation and differentiation. These cells produce a large amount of collagen, differentiate into osteoblasts, and thereafter form bones [29-31].

In osteoblasts, the expression of differentiation markers, such as collagen type I (Col-I), alkaline phosphatase (Alp), osteocalcin (Bglap), and runt-related transcription factor 2 (Runx2), increase depending on the extent of differentiation. Osteoblasts in the late stage of differentiation
produce mineralized deposits (calcified nodules) that stain with alizarin red [32, 33]. To the best of our knowledge, this study is the first to demonstrate the health effects of BCE on osteoblast differentiation.

**Materials and Methods**

**Materials and cell culture**

BCE powdered extract was CaNZac-35 (KOYO Mercantile Co., Tokyo, Japan), containing high-concentration polyphenols and anthocyanin (37.6 and 38.0% w/w, respectively) [10]. E2 (17β-estradiol) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The mouse pre-osteoblast cell line MC3T3-E1 was obtained from the Health Science Research Resources Bank, Osaka, Japan. It was maintained in α-minimum essential medium (α-MEM) (FUJI FILM Wako, Tokyo, Japan) with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (FUJIFILM Wako). Cell culture experiments were conducted at 37 °C in a humidified incubator containing 5% CO₂.

**Cell proliferation**

MC3T3-E1 cells (1 × 10⁴ per well) were seeded in 96-well plates and cultured overnight in α-MEM with 10% (v/v) FBS. This medium was replaced with a phenol red-free α-MEM, including 5% (v/v) charcoal-stripped FBS (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured for 48 h with BCE or 10nM E2 prior to quantification of their proliferation using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), according to the manufacturer’s protocol. The CCK-8 reagent was added to cells and incubated for 3 h at 37 °C. 450 nm absorbance was measured using a Benchmark microplate reader (Bio-Rad, Hercules, CA).
**Alkaline phosphatase assay**

MC3T3-E1 cells (5 × 10^4 per well) were seeded in 24-well plates and cultivated as described above. The growth medium was replaced with phenol red-free α-MEM containing 5 % (v/v) charcoal stripped FBS. Then, cells were cultured for 72 h with BCE or 10 nM E2. Cells were washed with 0.1 M Tris-HCl buffer (pH 9.8). Next, 100 μL of 0.1% (v/v) Triton X-100 in 0.1 M Tris-HCl buffer (pH 9.8) was added to the medium; plates were stored at -80 °C. Later, plates were rapidly thawed at 37 °C and assayed using the LabAssay™ ALP kit (FUJIFILM Wako). Protein concentrations were determined using a TaKaRa BCA Protein Assay Kit (TaKaRa Bio, Otsu, Japan). Absorbance relative to alkaline phosphatase activity and protein concentrations were measured on a Benchmark microplate reader (Bio-Rad) at wavelengths of 405 nm and 570 nm, respectively.

**Quantification of total collagen**

MC3T3-E1 cells (1 × 10^5 per well) were seeded in 12-well plates and cultivated overnight in α-MEM with 10 % (v/v) FBS. Then, the growth medium was replaced with differentiation medium (phenol red-free α-MEM containing 5 % [v/v] charcoal stripped FBS with Osteoblast-Inducer Reagent [TaKaRa Bio]). Next, the cells were cultured with BCE or 10 nM E2. The medium was replaced once every 3 days. Staining of collagen was performed on day 14 using a Sirius Red/Fast Green Collagen Staining Kit (Iwai Chemicals Co., Ltd., Tokyo, Japan) according to the manufacturer’s instructions. After treatment, cells were washed with PBS, followed by the addition of 0.5 mL of Kahle fixative for 10 min. Dye solution was added to the culture plates and incubated at 22 °C for 30 min. Cells were repeatedly rinsed with 0.5 mL of distilled water until the solution was colorless. Digital images were acquired using an Anyty™ digital microscope camera (3R-DKMCO4; 3R solution, Fukuoka, Japan). Following microscopy, the dye was eluted.
The optical density (OD) of the eluted dye solution was measured at 540 nm and 605 nm using a spectrophotometer (U-5100; HITACHI, Tokyo, Japan). The calculation formulas were: collagen (μg/section) = OD_{540} - (OD_{605} × 0.291)/0.0378; non-collagen proteins (μg/section) = OD_{605}/0.00204.

Mineralization

Calcified nodule formation was assessed using a calcification evaluation set (PG research, Tokyo, Japan) according to the manufacturer’s instructions. Briefly, 1 × 10^5 cells/well were seeded in 12-well plates in differentiation medium (Osteoblast-Inducer Reagent). Cells were treated on day 21 and the medium was replaced every 3 days. At the end of treatment, the medium was removed and the wells were washed with PBS. Cells were fixed at 22 °C for 10 min with neutral buffered formalin. The fixative was removed and the plate was washed with distilled water. Next, 1.0 mL of alizarin red solution was added, followed by incubation at 22 °C for 30 min. Micrographs were acquired using a fluorescence microscope FSX100 (OLYMPUS, Tokyo, Japan). After removing the water, 0.5 mL of calcified nodule lysate (5% [v/v] formic acid) was added, and the plate was stirred for 10 min to elute the dye. The absorbance of the eluate was measured at 450 nm using a spectrophotometer U-5100.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was prepared using the RNeasy mini kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (TaKaRa Bio). Expression levels of Col-I, Alp, Bglap, and Runx2 mRNAs were quantified via qPCR using TB Green Premix Ex Taq II (Tli RNaseH Plus; TaKaRa Bio). The PCR amplification protocol consisted of 5 s at 94 °C and 30 s at 60 °C for 40 cycles. Transcription
levels were normalized using those of actin beta (Actb). Primers were (5′→3′): Col-I, forward GAGCGGAGTACTGGATCG and reverse GCTTCTTTTCTTGGGGTT [34]; Alp, forward GATCATTCCCACGTTTTCACATT and reverse TTCACCGTCCACCACCTTTGT [34]; Bglap, forward GCGCTCTGTCTCTCTGACCT and reverse AAGCAGGGTCAAGCTCACAT [34]; Runx2, forward AGCGGCAGAATGGATGAGTC and reverse ACCAGACAACACCTTTTGACG [35]; and Actb, forward CATCCGTAAGACCTCTATGCCAAC and reverse ATGGAGCCACCGATCCACA [36]. PCR specificity was checked using melting curve analysis. All samples were analyzed in duplicate and relative gene expression was calculated using the 2\(^{-\Delta\Delta^Ct}\) method [37].

Animals and treatments

Ovariectomized (OVX) and sham-operated control female Sprague Dawley rats (CLEA Japan, Inc., Tokyo, Japan), aged 12 weeks, were housed in plastic cages in air-conditioned rooms with a 12-h light/dark cycle at the Institute for Animal Experiments of Hirosaki University Graduate School of Medicine. This study was approved by the Animal Research Committee of Hirosaki University (approval number G16004), in accordance with the rules for animal experimentation of Hirosaki University. Our previous study showed that 3% BCE has phytoestrogenic effects in rats [10]. Thus, as this is a continuous study, the rat model used here is the same as that in the previous study. All rats received the AIN-93M diet, with and without 3% BCE, as indicated (Oriental Yeast Co., Ltd., Tokyo, Japan) and were divided into three groups: 1) sham surgery without BCE treatment, 2) OVX without BCE treatment (control), and 3) OVX treated with 3% BCE for three months. All rats had free access to water and food. At the end of the experiment, rats were euthanized and femurs were excised and fixed in 10% (v/v) formaldehyde, demineralized in 10% (v/v) EDTA-2Na (pH 7.2) at 4 °C for three weeks, and embedded in paraffin.
Femur sections (4 μm) were routinely passed through xylene and a graded alcohol series before alkaline phosphatase staining.

**Alkaline phosphatase staining**

To measure femoral alkaline phosphatase activity, femurs were stained with a TRAP/ALP kit (FUJIFILM Wako) according to the manufacturer’s instructions, and nuclei were stained with hematoxylin. Specimens were examined and photographed using a fluorescence microscope FSX100.

**Statistical analysis**

Results are expressed as the mean ± standard deviation (SD). Graphs were generated using GraphPad Prism version 7.03 software (GraphPad, San Diego, CA, USA). Statistically significant differences were determined using Kruskal–Wallis analysis with the Steel post-hoc test using Bell Curve for Excel version 3.2 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Results with *p*-values <0.05 were considered statistically significant.

**Results**

**MC3T3-E1 proliferation**

Cells were treated with multiple concentrations of BCE that had phytoestrogenic effects in our previous studies [12, 13] or 10 nM E2 as a positive control, and cultured for 48 h. No significant differences were observed in proliferation following treatment with 0.2, 1.0, or 5.0 μg/mL BCE or E2. However, proliferation was significantly decreased after treatment with 20.0 μg/mL BCE (*P < 0.05*) (Fig. 1).
**Alkaline phosphatase**

MC3T3-E1 cells were treated with multiple concentrations of BCE or 10 nM E2 and cultured for 72 h. Alkaline phosphatase activity significantly increased in a dose-dependent manner up to 5.0 μg/mL BCE and E2 ($P < 0.05$) (Fig. 2).

**Total collagen**

MC3T3-E1 cells were treated with multiple concentrations of BCE or 10 nM E2 and cultured for 14 days. Total collagen was quantified via Sirius Red staining. The collagen content increased significantly after treatment with all concentrations of BCE and 10 nM E2 ($P < 0.05$) (Fig. 3a and b).

**Osteogenic marker expression**

MC3T3-E1 cells were incubated with multiple concentrations of BCE or 10 nM E2 for 24 h. Bone differentiation marker expression was measured using RT-qPCR. *Col-I, Alp, Bglap*, and *Runx2* expression significantly increased after treatment with all concentrations of BCE and 10 nM E2 ($P < 0.05$) (Fig. 4).

**Mineralization**

MC3T3-E1 cells were treated with multiple concentrations of BCE or 10 nM E2 and cultured for 21 days. Cells were stained with alizarin red to assess their degree of mineralization, which revealed the presence of calcified nodules (Fig. 5a). The levels of calcified nodules significantly increased after treatment with all concentrations of BCE and 10 nM E2 ($P < 0.05$) (Fig. 5b).
Discussion

A previous study reported that BCE inhibits osteoclast differentiation [28]; however, its effects on osteoblasts are unknown. The present study focused on the differentiation of MC3T3-E1 pre-osteoblasts into osteoblasts after treatment with BCE.

Some studies have reported that the administration of 10 nM estrogen significantly enhances MC3T3-E1 cell proliferation [29, 38]. However, this was not observed in this study. Specifically, the administration of 10 nM E2 did not increase proliferation, whereas BCE and E2 did slightly, although not significantly. A previous study also reported no difference in osteoblast proliferation after the administration of E2 or phytoestrogen, which is consistent with our results [31]. Additionally, proliferation significantly decreased after treatment with 20 µg/mL BCE, suggesting that concentrations equal or greater than this are toxic to MC3T3-E1 cells.

MC3T3-E1 cells have been shown to increase alkaline phosphatase activity and collagen secretion due to estrogen action. Here, BCE and E2 increased alkaline phosphatase activity (Fig. 2) and collagen secretion (Fig. 3) in MC3T3-E1 cells in a concentration-dependent manner. As Col-I, Alp, Bglap, and Runx2 are known osteoblast differentiation markers, the expression of all four genes was increased by BCE and E2, which suggests that this phenomenon is due to phytoestrogen activity.

There have been several reports of anthocyanin induction of osteoblast differentiation, with increased expression of collagen, alkaline phosphatase, osteocalcin, and Runx2, consistent with our findings [33, 39, 40]. Runx2 is a transcription factor required for osteoblast differentiation and is expressed at an early stage of differentiation [41]. In contrast, Col-I, Alp, and Bglap are the earliest biomarkers of mature osteoblastic differentiation [42, 43]. In addition to the increased expression of these genes (Fig. 4), increased alkaline phosphatase activity and secretion of collagen at the protein level were observed (Figs. 2 and 3). Furthermore, an increase in the levels
of calcified nodules was detected (Fig. 5). These results suggest that BCE induces the differentiation of pre-osteoblasts into osteoblasts and osteogenesis.

Whether osteoblast differentiation was induced in vivo was briefly investigated. OVX rats were used as menopausal models to evaluate the promotion of osteogenic differentiation of femoral tissue. Alkaline phosphatase staining has been shown to be strong in the region surrounding the epiphysis because active osteoblast differentiation occurs there [44]. The femurs of sham rats, OVX rats without BCE treatment (control), and OVX rats treated with 3% BCE were stained for alkaline phosphatase. The staining intensity of epiphyses of control rats was weaker than that of those of sham-operated rats. However, the staining intensity of epiphyses from OVX rats treated with 3% BCE was stronger than that of those from control rats (Supplementary Fig. S1). BCE is reportedly effective in animal osteoporosis models [24, 25]. However, to our knowledge, there has been no study of osteoblast differentiation in vivo, and this study clarifies that. Still, since our in vivo experiment was simple, more detailed studies are required.

Moriwaki et al. reported that bilberry, BCE, and their anthocyanins have a preventive effect on osteoporosis mediated by the inhibition of excessive osteoclastogenesis [28]. Although osteoblasts were also used in their study, the administration of BCE caused no change in cell differentiation [28]. Although there have been reports showing that estrogen and phytoestrogen enhance osteoblast differentiation, results may differ depending on the estrogen sensitivity of the osteoblasts. In this study, it was found that phytoestrogen effects are more likely to appear with estrogen-sensitive cells such as MC3T3-E1 cells.

Polyphenols include anthocyanin-rich plants and are known to attenuate osteoporosis [25, 28]. Furthermore, their mechanism is reported to be 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 [45, 46]. This study focused on phytoestrogen activity, and previous
studies suggest that pathways other than estrogen signaling pathways are also activated. However, the mechanism is complex, as phytoestrogens activate the signal transducers and activators of transcription PI3K-AKT, Src/ERK1/2, and nuclear factor-kappa B through estrogen receptors within seconds [47, 48]. Moreover, BCE likely interacts with these factors through estrogen receptors. From microarrays using breast cancer MCF7 cells with high expression of estrogen receptors, BCE activated multiple pathways [10].

During menopause, women are likely to develop osteoporosis owing to decreased estrogen secretion. Although hormone replacement therapy is available for the treatment of osteoporosis, it is associated with several problems, including increased risk of breast cancer and side effects [49, 50].

There are many studies showing that the intake of phytoestrogens, such as soy isoflavones and equol, promotes the prevention and amelioration of osteoporosis [22, 23]. However, as phytoestrogens are not suitable for people who are allergic to soybeans, the effects of blackcurrant on osteoporosis are of great importance. Nevertheless, future trials testing its effects human are required to validate our findings.

Conclusion

BCE and E2 did not induce cell proliferation but increased alkaline phosphatase activity and collagen production. They increased the expression of markers for osteocyte differentiation and the number of alizarin-positive cells, promoting differentiation into osteoblasts. Thus, although BCE was already known to have phytoestrogenic effects, our data suggest that it also affects osteoblast differentiation. Further testing is necessary; yet, the results of this study raise expectations that BCE could reduce osteoporosis during menopause.
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Statements and Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

NN and KH designed the study; NN and KH performed experiments and analyzed data; NN wrote the original manuscript; IO edited the manuscript. All authors reviewed and approved the final manuscript for publication.

Ethics Approval

This study was approved by the Animal Research Committee of Hiroaki University (approval number G16004).

Data Availability

The data generated in the present study is available from the corresponding author upon request.
Figure legends

Fig. 1 MC3T3-E1 cell proliferation assay.
Blackcurrant extract (BCE) does not significantly increase MC3T3-E1 proliferation. Data shown as means ± standard deviation from six independent experiments normalized to control cell counts. *P < 0.05 vs. Control.

Fig. 2. Effect of blackcurrant extract (BCE) treatment on alkaline phosphatase activity in MC3T3-E1 cells.
BCE treatment increases alkaline phosphatase activity in MC3T3-E1 cells. Data shown as means ± standard deviation from four independent experiments. *P < 0.05 vs. Control.

Fig. 3. Effect of blackcurrant extract (BCE) treatment on collagen production in MC3T3-E1 cells.
(a) MC3T3-E1 cells were treated with the indicated concentrations of BCE or 10 nM 17β-estradiol (E2) for 14 days. The cells were stained with a Sirius Red/Fast Green Collagen Staining Kit. Collagen was stained red. Scale bar: 500 μm. (b) Pigment was extracted and measured via spectrophotometry. Data shown as means ± standard deviation from four independent experiments. *P < 0.05 vs. Control.

Fig. 4. Expression of osteogenesis marker genes in MC3T3-E1 cells.
Blackcurrant extract (BCE) and 17β-estradiol (E2) increase expression of osteogenesis marker genes. MC3T3-E1 cells were treated with the indicated concentrations of BCE or 10 nM E2 for 24 h. Relative expression was normalized to that of Actb. Data shown as means ± standard deviation from four independent experiments. *P < 0.05 vs. Control.
Fig. 5. Effect of blackcurrant extract (BCE) treatment on the mineralization of MC3T3-E1 cells.

(a) MC3T3-E1 cells were treated with the indicated concentrations of BCE or 10 nM 17β-estradiol (E2) for 21 days. The cells were stained with a mineralization evaluation set. Mineralization areas were stained red. Scale bar: 500 μm. (b) Pigment was extracted with calcified nodule lysate (5% [v/v] formic acid) and measured using a spectrophotometer. Data shown as means ± standard deviation from four independent experiments. *P < 0.05 vs. Control.

Supplementary Material

Supplementary Fig. S1. Light micrographs of alkaline phosphatase staining of the femur.

Representative alkaline phosphatase staining images of the femur sections of (a) sham-operated, (b) ovariectomized (OVX) + 0% blackcurrant extract (BCE), and (c) OVX + 3% BCE rats. Scale bar: 200 μm.
Figure 1

MC3T3-E1 cell proliferation assay.

Blackcurrant extract (BCE) does not significantly increase MC3T3-E1 proliferation. Data shown as means ± standard deviation from six independent experiments normalized to control cell counts. *P < 0.05 vs. Control.
Figure 2

Effect of blackcurrant extract (BCE) treatment on alkaline phosphatase activity in MC3T3-E1 cells.

BCE treatment increases alkaline phosphatase activity in MC3T3-E1 cells. Data shown as means ± standard deviation from four independent experiments. *$P<0.05$ vs. Control.
Figure 3

Effect of blackcurrant extract (BCE) treatment on collagen production in MC3T3-E1 cells.

(a) MC3T3-E1 cells were treated with the indicated concentrations of BCE or 10 nM 17β-estradiol (E2) for 14 days. The cells were stained with a Sirius Red/Fast Green Collagen Staining Kit. Collagen was stained red. Scale bar: 500 μm. (b) Pigment was extracted and measured via spectrophotometry. Data shown as means ± standard deviation from four independent experiments. *P< 0.05 vs. Control.
Expression of osteogenesis marker genes in MC3T3-E1 cells.

Blackcurrant extract (BCE) and 17β-estradiol (E2) increase expression of osteogenesis marker genes. MC3T3-E1 cells were treated with the indicated concentrations of BCE or 10 nM E2 for 24 h. Relative expression was normalized to that of Actb. Data shown as means ± standard deviation from four independent experiments. *P < 0.05 vs. Control.
Figure 5

Effect of blackcurrant extract (BCE) treatment on the mineralization of MC3T3-E1 cells.

(a) MC3T3-E1 cells were treated with the indicated concentrations of BCE or 10 nM 17β-estradiol (E2) for 21 days. The cells were stained with a mineralization evaluation set. Mineralization areas were stained red. Scale bar: 500 μm. (b) Pigment was extracted with calcified nodule lysate (5% [v/v] formic acid) and measured using a spectrophotometer. Data shown as means ± standard deviation from four independent experiments. *P < 0.05 vs. Control.

Supplementary Files

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