Original Research

Arbutin ameliorates glucocorticoid-induced osteoporosis through activating autophagy in osteoblasts

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Impact statement

Glucocorticoid-related osteoporosis, an adverse event of long-term glucocorticoid treatment, is a significant major public health problem. Therefore, development of effective treatments for this disease is of utmost importance. Our results provide evidence of the protective effects of arbutin (a compound derived from natural products) on glucocorticoid-induced osteoporosis. These effects are associated with increased differentiation and mineralization of osteoblasts via autophagy activation. This study highlights that arbutin has protective effects on glucocorticoid-related osteoporosis.

Abstract

Chronic long-term glucocorticoid use causes osteoporosis partly by interrupting osteoblast homeostasis and exacerbating bone loss. Arbutin, a natural hydroquinone glycoside, has been reported to have biological activities related to the differentiation of osteoblasts and osteoclasts. However, the role and underlying mechanism of arbutin in glucocorticoid-induced osteoporosis are elusive. In this study, we demonstrated that arbutin administration ameliorated osteoporotic disorders in glucocorticoid dexamethasone (Dex)-induced mouse model, including attenuating the loss of bone mass and trabecular microstructure, promoting bone formation, suppressing bone resorption, and activating autophagy in bone tissues. Furthermore, Dex-stimulated mouse osteoblastic MC3T3-E1 cells were treated with arbutin. Arbutin treatment rescued Dex-induced repression of osteoblast differentiation and mineralization, the downregulation of osteogenic gene expression, reduced autophagic marker expression, and decreased autophagic puncta formation. The application of autophagy inhibitor 3-MA decreased autophagy, differentiation, and mineralization of MC3T3-E1 cells triggered by arbutin. Taken together, our findings suggest that arbutin treatment fends off glucocorticoid-induced osteoporosis, partly through promoting differentiation and mineralization of osteoblasts by autophagy activation.

Keywords: Arbutin, dexamethasone, osteoporosis, osteoblasts, autophagy

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Introduction

Osteoporosis is a progressive systemic skeletal disease characterized by osteopenia and deterioration of bone microstructure, which results in bone fragility and increased risk of fracture.[1] As the incidence of osteoporotic fracture increases, the resulting disability and mortality increases, representing a huge burden to society worldwide.[1] Glucocorticoid is commonly employed to treat inflammatory and immune-mediated diseases, such as allergy, rheumatoid arthritis, and leukemia.[2] Glucocorticoid-related osteoporosis is the most devastating adverse event of glucocorticoid treatment.[3] Chronic glucocorticoid use reduces bone formation and facilitates bone resorption by decreasing the number and activity of osteoblasts and by prolonging the lifespan of osteoclasts.[4] Thus, the development of effective therapeutic approaches is of utmost importance to mitigate glucocorticoid-induced osteoporosis.

Autophagy is a homeostatic process involving autophagosome formation, the sequestration of cellular components in autophagosomes, autophagosome-lysosome fusion, and lysosomal degradation of cellular components. It removes abandoned organelles or proteins to maintain the stability of the intracellular environment.[5] Autophagy plays a variety of roles in human physiology and is also implicated in the pathophysiological processes of diseases.[6] With respect to the effect of autophagy on bone metabolism, autophagy has been demonstrated to maintain bone homeostasis, and dysregulation of this pathway results in osteoporosis. Autophagy maintains osteoblast viability under stressful conditions[7–9] and is an important regulator in the differentiation and mineralization of osteoblasts.[10–12]

Moreover, it has been shown that glucocorticoid has an inhibitory effect on osteoblast autophagy, which is implicated in the pathogenic mechanism of glucocorticoid-induced osteoporosis.[12–14]

Arbutin is a natural hydroquinone glycoside which is found in various plants, including Vaccinium, Asteraceae, and Ericaceae.[15] Arbutin possesses various biological properties, such as skin whitening, and has hypoglycemic, neuroprotective, anti-inflammatory, antioxidant, and anti-tumor properties.[16–21] The role of arbutin in bone homeostasis has been documented both in osteoclasts and osteoblasts. Arbutin inhibits osteoclast differentiation via the suppression of superoxide production and downregulation of osteoclastogenesis regulator NFATc1.[22] As regards osteoblasts, arbutin facilitates proliferation and differentiation of MC3T3-E1 cells by modulating Wnt/β-catenin signaling.[23] Additionally, it has been demonstrated that arbutin protects human neuroblastoma cells from apoptosis by regulating AMP-activated protein kinase (AMPK)-p62, which is involved in the autophagy pathway.[24] Considering autophagy is implicated in glucocorticoid-induced osteoporosis, it would be worthwhile investigating whether arbutin could affect glucocorticoid-induced osteoporosis via the modulation of autophagy.

In the current study, the effects of arbutin on bone loss, bone formation, bone resorption, and autophagy were studied using an osteoporotic mice model induced by dexamethasone (Dex, a synthetic glucocorticoid). In addition, we explored the regulatory effects of arbutin on differentiation, matrix mineralization, and autophagy of Dex-treated mouse osteoblastic MC3T3-E1 cells. Our research ascertained that arbutin promoted differentiation and mineralization of osteoblasts via autophagy activation, thereby alleviating glucocorticoid-induced osteoporosis.

Materials and methods

Animals and treatments

Male C57BL/6J mice (six-week-old) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. All mice were housed under specified pathogen free (SPF) environment (temperature, 22 ± 1°C; humidity, 45–55%; light/dark cycle, 12 h). Mice were randomly divided into four groups ($n = 6$ for each group): (1) control group; (2) Dex group; (3) Dex + 100 mg/kg arbutin group; and (4) Dex + 200 mg/kg arbutin group. For the osteoporosis model construction, mice were intraperitoneally injected with Dex (50 mg/kg; Aladdin, D137736) once daily for five weeks. Simultaneously, arbutin treatment (100 mg/kg or 200 mg/kg; Aladdin, A106856) via intraperitoneal injection was administered once daily and continued for five weeks. The control group received intraperitoneal injection of normal saline. At the endpoint, blood samples were collected. After euthanasia, femora were excised for subsequent experiments. The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of China Medical University (Ethical number: 2019PS555K).

Microcomputed tomography analysis

Femora were fixed with 4% paraformaldehyde and scanned using a microcomputed tomography (microCT) system (QuantumGX, PerkinElmer, USA). The parameters used were as follows: X-ray intensity, 50 kVp, 200 μA; pixel size, 14.85 μm; AI filter, 0.5 mm. Using cone beam reconstruction software with a Feldkamp-based algorithm, the dataset was reconstructed and then segmented into two-dimensional images by adaptive thresholding. After obtaining 200 tomographic sections, three-dimensional surface-rendered models were generated using CTan software. Measurements were made of bone mineral density (BMD), trabecular bone thickness (Tb.Th), trabecular bone numbers (Tb.N), trabecular spacing (Tb.Sp), and bone volume/tissue volume (BV/TV).

Hematoxylin and eosin staining

Femora were fixed, decalcified, and embedded in paraffin. After being sliced longitudinally into 5 μm sections, the sections were stained with hematoxylin and eosin (HE). The images were captured (at ×40 magnification), and the trabecular area was quantified.

Measurement of cross-linked C-telopeptide of type I collagen, tartrate-resistant acid phosphatase, osteocalcin, and alkaline phosphatase

The levels of cross-linked C-telopeptide of type I collagen (CTX-1) and osteocalcin in serum were determined by enzyme-linked immunosorbent assay (ELISA) Kits from Uscn Life Science Inc. (Cat. No. CEA665Mu, SEA471Mu). Tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) activities in serum were measured with Kits from Nanjing Jiancheng Bioengineering Institute (Cat. No. A058, A059). All experiments were conducted according to the respective manufacturer's instructions. To detect ALP activity in cell samples, cells were subjected to ultrasonication in phosphate-buffered saline (PBS). After centrifugation, the supernatants were collected to measure protein concentration. ALP activity was normalized to protein concentration.

Western blot analysis

Femora were lysed with radioimmunoprecipitation assay (RIPA) buffer (Solarbio, R0010). Proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred into polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk, followed by incubation with primary antibodies against Runx2 (Abclonal, A2851; 1:1000), BMP2 (affinity, AF5163; 1:500), p62 (CST, #5114; 1:1000), Beclin 1 (CST, #3495; 1:1000), Atg7 (affinity, DF6130; 1:500), and LC3 (affinity, AF5402; 1:500) overnight. After washing in Tris buffer saline Tween-20 (TBST) buffer, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (Solarbio, SE134, SE131; 1:3000). Protein blots were visualized with electrochemiluminescence (ECL) Western blotting substrate (Solarbio, PE0010) and analyzed by Gel-Pro-Analyzer.

Cell culture and treatment

Mouse osteoblastic MC3T3-E1 cells were purchased from iCell Bioscience Inc and incubated in minimum essential medium (MEM) α medium supplemented with 10% FBS. For the induction of osteoblast differentiation, MC3T3-E1 cells were cultured in osteogenic medium (MEM α medium, 10% FBS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid) with 1 μM Dex, as previously described.[25] Simultaneously, 100 μM of arbutin was added in the osteogenic medium. After treatment for three days, cells were collected to detect autophagy activation via Western blot analysis. The levels of osteogenic markers and ALP activity were determined after treatment for seven days. To estimate the effect of arbutin on autophagy, MC3T3-E1 cells were maintained in osteogenic medium containing Dex (1 μM) and arbutin (100 μM) in the presence or absence of 3-methyladenine (3-MA) (1 mM).[26]

Alizarin red staining

MC3T3-E1 cells were subjected to different treatments for 18 days. Subsequently, the culture medium was discarded. Cells were stained with Alizarin red solution (Shanghai Yuanye Bio-Technology Co., Ltd, Cat. No. S19145). After washing with PBS, a panoramic image was photographed, and matrix mineralization was visualized using a microscope (at ×100 magnification).

Analysis of autophagy by GFP–LC3 puncta

MC3T3-E1 cells were transfected with pBABEpuro GFP-LC3 plasmid (Addgene, USA) using Lipofectamine 2000 (Invitrogen, USA). After 24 h, the medium was replaced with osteogenic medium containing 1 μM Dex and 100 μM of arbutin. Following treatment for three days, the GFP signal was observed by microscopy (at ×400 magnification). Cells containing GFP-LC3 puncta were considered to be autophagy-positive.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.0 (GraphPad Software, USA). The results are presented as the mean ± standard deviation. Comparison among multiple groups was performed by one-way ANOVA followed by Tukey's *post hoc* test. $P < 0.05$ was considered to have statistical significance.

Results

Arbutin alleviated Dex-induced bone loss and bone deterioration

To explore whether arbutin has bioactivity on antiosteoporosis, Dex-induced osteoporotic mice were treated with 100 mg/kg or 200 mg/kg arbutin. 3D images of microCT and trabecular parameters showed that Dex induced sparse trabecular microstructure, reduced BMD, Tb.Th, Tb.N, and BV/TV, as well as increased Tb.Sp. Arbutin administration lessened the severity of Dex-induced bone degradation, as evidenced by the restoration of mineral density, trabecular thickness, trabecular number, and trabecular bone volume and reduced trabecular separation (Figure 1a and b). As shown by HE staining, reduced trabecular area and increased Tb.Sp were observed in Dex-induced mice in contrast to the control group, while arbutin treatment increased trabecular area and reduced Tb.Sp in the distal femur (Figure 1c). The results demonstrated that arbutin could alleviate bone loss and bone deterioration of Dex-induced mice.

Effect of arbutin on biochemical markers of bone turnover

Next, bone resorption and formation in the bony tissue of different groups were evaluated. CTX-1 level and TRAP activity in serum were upregulated in Dex-treated mice. In contrast, the expression of these bone resorption markers was blocked in response to arbutin treatment (Figure 2a and b). Meanwhile, serum osteocalcin level and ALP activity in Dex-treated mice dropped, whereas arbutin treatment rescued Dex-induced downregulation of bone formation markers (Figure 2c and d). The levels of osteogenic differentiation markers including Runx2 and BMP2 were further determined via Western blot analysis. Arbutin administration attenuated Dex-induced downregulation of Runx2 and BMP2 in the femur of mice (Figure 2e and f). These data indicated that arbutin facilitated bone formation and suppressed bone resorption of Dex-induced mice.

Arbutin mitigated the inhibitory effects of Dex on autophagy in femur of osteoporotic mice

To further explore the regulatory mechanism of arbutin on Dex-induced osteoporosis, the impact of arbutin on autophagy in the femur was examined. The levels of Beclin 1, Atg7, and LC3II/I in the femur from Dex-induced mice were reduced, and p62 expression was increased, indicating that autophagy was suppressed by Dex. Arbutin intervention activated autophagy in the femur of Dex-induced mice, as evidenced by the increased expression of Beclin 1, Atg7, and LC3II/I and reduced p62 expression (Figure 3a and b). These results demonstrated that arbutin mitigated the suppressive effects of Dex on autophagy in the femur of osteoporotic mice.

Arbutin promoted osteogenic differentiation and mineralization of Dex-treated MC3T3-E1 cells

Previously, we found that 100 μM arbutin promoted osteoblastic MC3T3-E1 cell proliferation and differentiation.[23] In the present study, we further identified the effects of 100 μM arbutin on Dex-treated MC3T3-E1 cells. Following treatment of MC3T3-E1 cells with Dex and arbutin for seven days, the levels of Runx2 and BMP2 in the cells were examined via Western blot analysis. The results showed that Dex treatment suppressed the expression of Runx2 and BMP2, while arbutin treatment exhibited a restored expression for these proteins (Figure 4a and b). Meanwhile, ALP activity was analyzed to evaluate osteogenic differentiation. Dex restrained ALP activity in MC3T3-E1 cells, and arbutin mitigated the suppressive

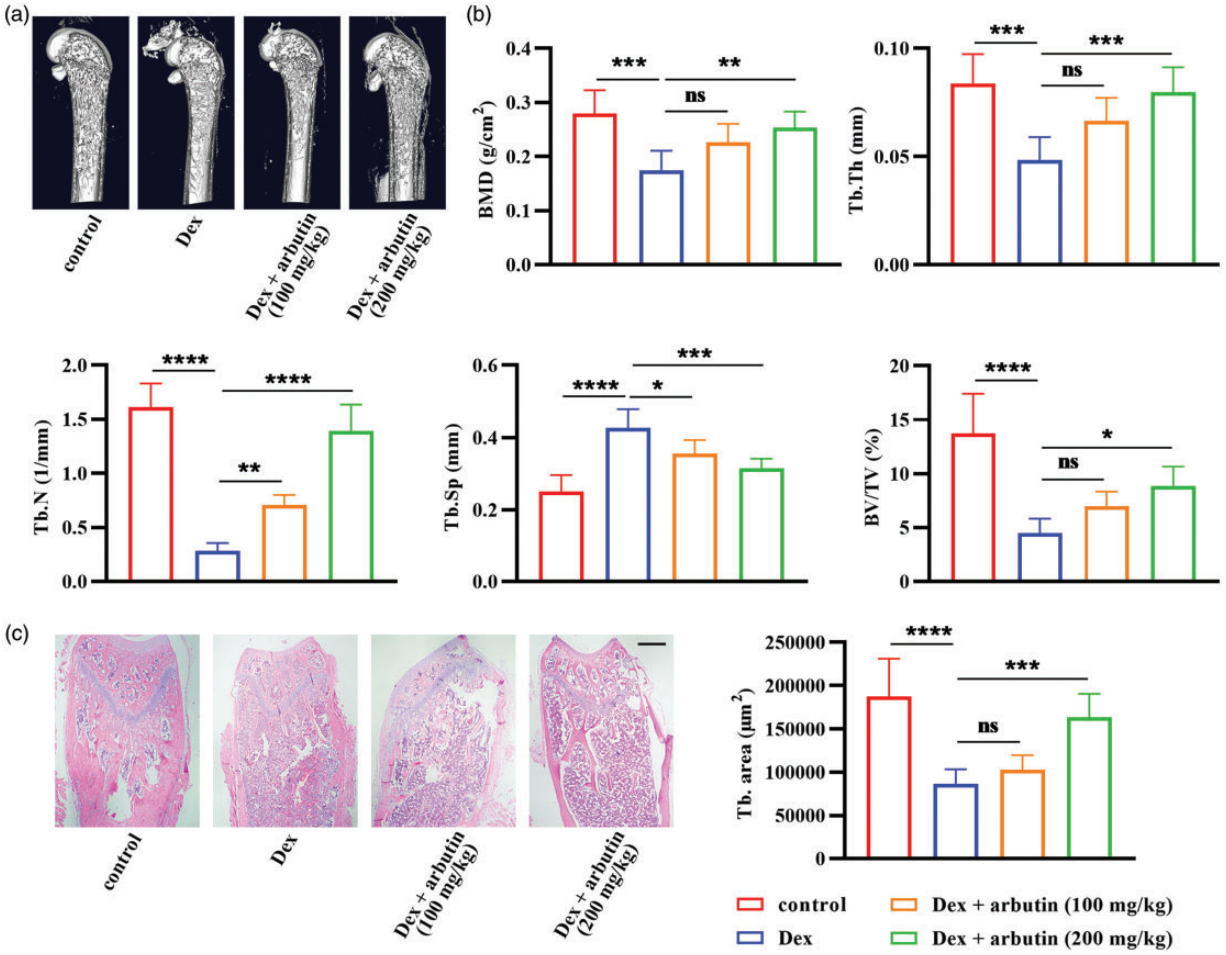

Figure 1. Arbutin alleviated Dex-induced bone loss and bone deterioration. (a) MicroCT images of femora in different experimental groups. (b) Trabecular parameters calculated from microCT included BMD, Tb.Th, Tb.N, Tb.Sp, and BV/TV. (c) Histological changes in femur exhibited by HE staining and quantification of trabecular area. Scale bar, 500 μm. All data are represented as mean ± SD ($n = 6$/group). *P<0.05, **P<0. 01, ***P<0.001, ****P<0.0001. (A color version of this figure is available in the online journal.)
BMD: bone mineral density; Tb.Th: trabecular bone thickness; Tb.N: trabecular bone numbers; Tb.Sp: trabecular spacing; BV/TV: bone volume/tissue volume.

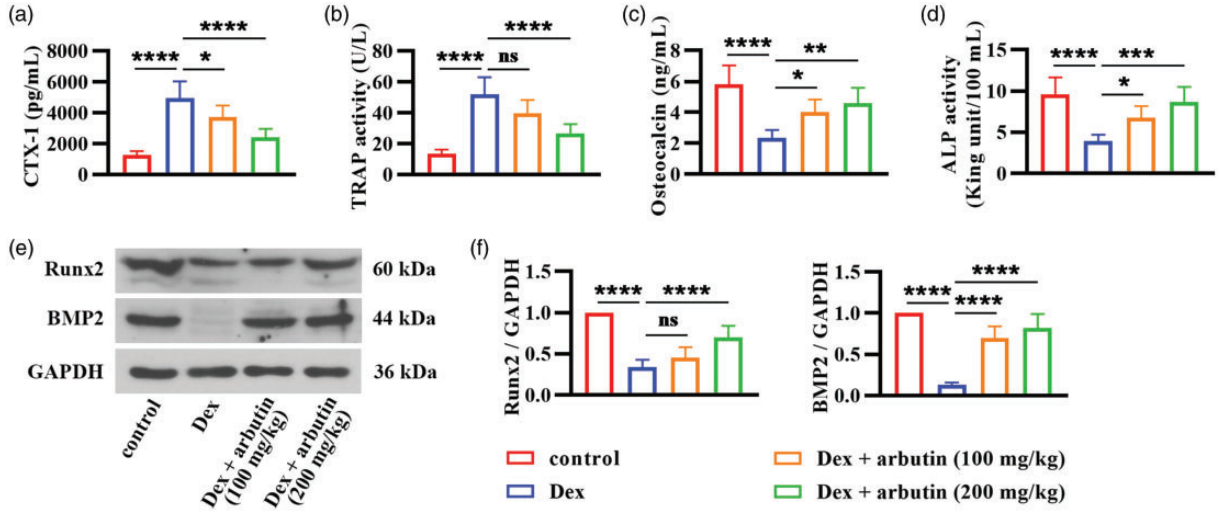

Figure 2. Effect of arbutin on biochemical markers of bone turnover. (a, b) Serum levels of bone resorption markers: CTX-1 and TRAP. (c, d) Serum levels of bone formation markers: osteocalcin and ALP. (e, f) Femoral Runx2 and BMP2 levels detected by Western blot analysis, and the relative expression to GAPDH was quantified. Data presented as mean ± SD ($n = 6$/group). *P<0.05, **P<0. 01, ***P<0.001, ****P<0.0001. (A color version of this figure is available in the online journal.)
CTX-1: cross-linked C-telopeptide of type I collagen; TRAP: tartrate-resistant acid phosphatase; ALP: alkaline phosphatase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

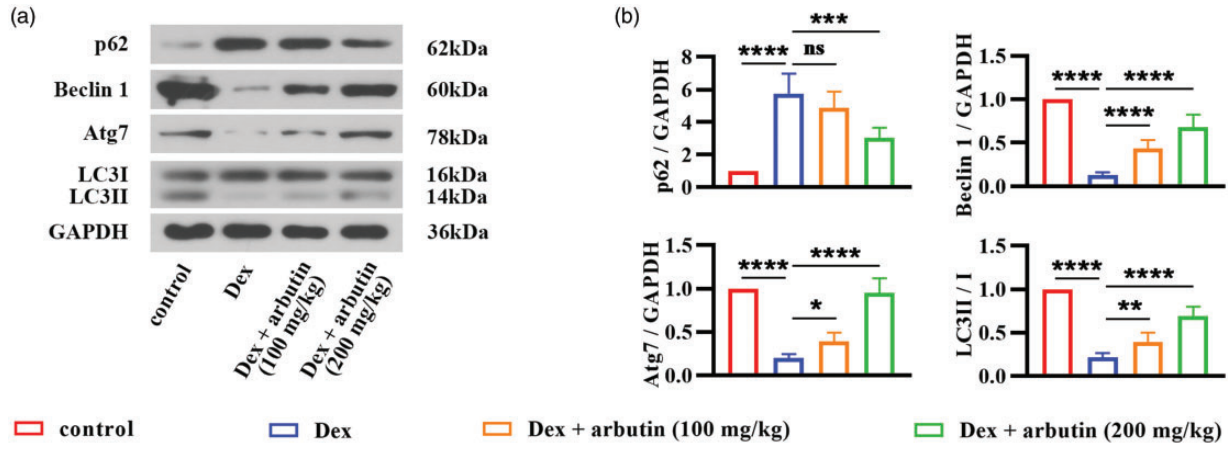

Figure 3. Arbutin mitigated the inhibitory effects of Dex on autophagy in femur of osteoporotic mice. (a) Western blot analysis was performed to determine the levels of autophagy-related proteins. (b) Quantitative analysis of relative expression. Data represented as mean ± SD ($n = 6$/group). *P<0.05, **P<0. 01, ***P<0.001, ****P<0.0001. (A color version of this figure is available in the online journal.)
GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

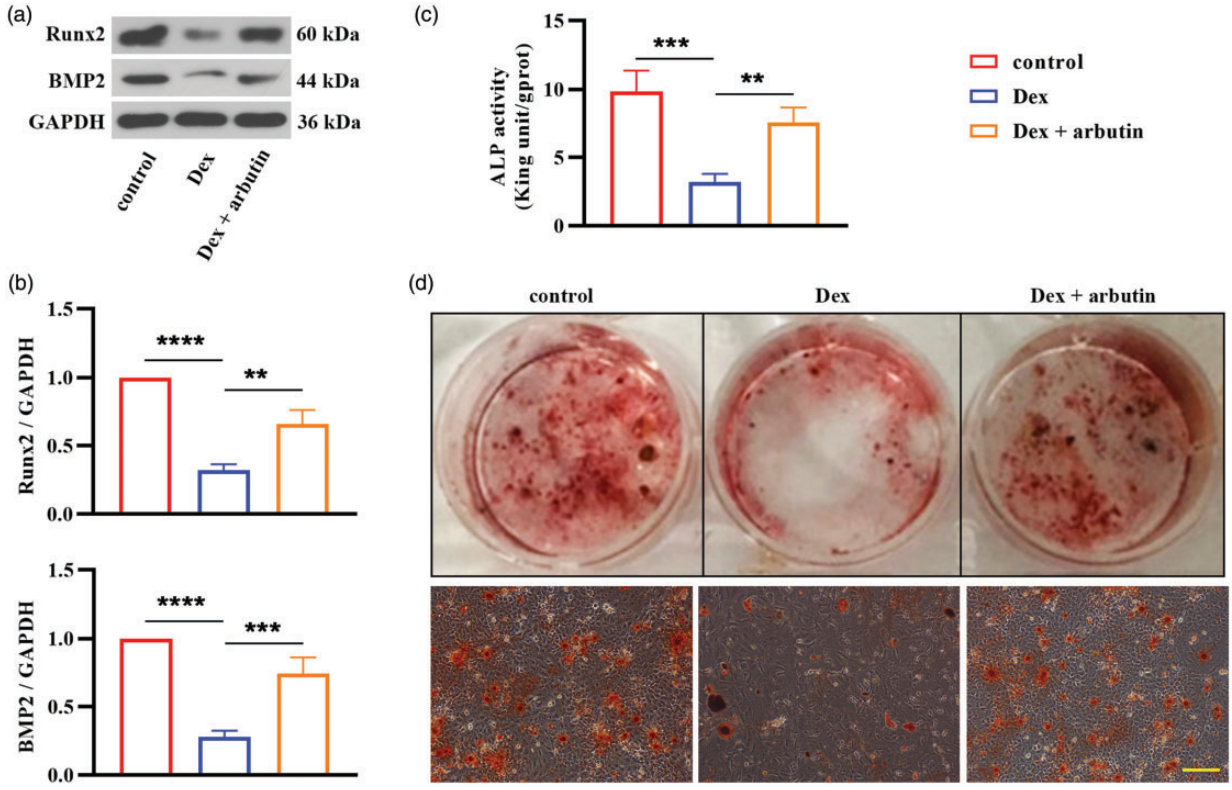

Figure 4. Arbutin promoted osteogenic differentiation and mineralization of Dex-treated MC3T3-E1 cells. Mouse osteoblast MC3T3-E1 cells were maintained in osteogenic medium containing 1 μM DEX and 100 μM arbutin. (a, b) Western blot analysis presented the protein levels of Runx2 and BMP2 of MC3T3-E1 cells on the seventh day. (c) ALP activity in MC3T3-E1 cells was assessed after 7 days of osteogenic induction. (d) Alizarin red staining after 18 days of osteogenic induction of MC3T3-E1 cells. Lower panel: matrix mineralization was visualized using a microscope. Scale bar, 200 μm. All data are represented as mean ± SD ($n = 3$/group).**P<0. 01, ***P<0.001, ****P<0.0001. (A color version of this figure is available in the online journal.)
GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ALP: alkaline phosphatase.

effect of Dex on ALP activity (Figure 4c). The result of Alizarin red staining showed that mineralization of Dex-treated cells was impaired, and treatment with arbutin led to increased osteogenic mineralization in MC3T3-E1 cells (Figure 4d). Thus, arbutin treatment ameliorated Dex-induced impairment of the osteogenic function of MC3T3-E1 cells.

Arbutin activated autophagy in Dex-stimulated MC3T3-E1 cells

To prove the role of arbutin in autophagy of MC3T3-E1 cells, we treated cells with Dex and arbutin for three days, followed by determining the levels of autophagy-related genes. The results indicated that Dex increased p62

expression and reduced the levels of Beclin 1, Atg7, and LC3II/I. However, arbutin treatment reversed the changes in the expression of these markers (Figure 5a and b). Moreover, autophagosome formation was observed by fluorescence microscopy. GFP-LC3 puncta formation was decreased in Dex-treated MC3T3-E1 cells, while arbutin treatment led to the increase of puncta-positive cells (Figure 5c). Collectively, these data suggested that arbutin triggered autophagy in Dex-stimulated MC3T3-E1 cells.

Arbutin facilitated differentiation and mineralization of Dex-stimulated MC3T3-E1 cells by activating autophagy

To verify whether arbutin improved osteogenic function via activating autophagy, MC3T3-E1 cells were treated with Dex and arbutin and with or without autophagy inhibitor 3-MA. Arbutin treatment resulted in downregulation of p62 and increased LC3-II/I level in Dex-stimulated MC3T3-E1 cells. Co-treatment with 3-MA reversed the pro-autophagic effects of arbutin, which was shown as increased p62 expression and reduced LC3-II/I level (Figure 6a and b). In addition, the protein expression of osteoblast marker was further detected by Western blot analysis. Arbutin upregulated Runx2 and BMP2 levels of Dex-stimulated MC3T3-E1 cells, whereas its pro-osteoblastic effect against Dex was abolished under 3-MA treatment (Figure 6c and d). Consistent with the above results, arbutin treatment facilitated osteoblast differentiation (Figure 6e) and mineralization function (Figure 6f). However, 3-MA treatment eliminated the promotion of arbutin on osteogenic differentiation and mineralization. The data revealed that arbutin had the pro-osteoblastic function in Dex-treated MC3T3-E1 cells through activating autophagy.

Discussion

Glucocorticoid-induced osteoporosis is attributable to its effects on diverse cell types in bone, including osteoblasts, osteocytes, and osteoclasts. Generally, the effect of glucocorticoid on osteoblasts is deemed the most important event in mediating bone loss in patients with long-term glucocorticoid treatment. Reduced bone formation in glucocorticoid-induced osteoporosis has been ascribed as follows: first, inhibition of osteoblast replication; second, repression of osteoblast differentiation; third, acceleration of osteoblast and osteocyte apoptosis.[3] Therefore, identifying potential pharmacological interventions to promote bone formation by osteoblasts may conduce prevention or treatment of glucocorticoid-induced osteoporosis. Our data showed that arbutin can alleviate Dex-induced osteoporosis in mice. Arbutin administration decreased bone loss, promoted bone formation, and suppressed bone resorption, concomitant with autophagy activation. Moreover, arbutin treatment increased differentiation and mineralization of Dex-induced osteoblasts, and it exerted this effect through activating autophagy. Our study preliminarily revealed the protective effect of arbutin treatment on glucocorticoid-induced osteoporotic mice and mouse osteoblasts. To propose arbutin as a potential drug for glucocorticoid-induced osteoporosis, long-term use of arbutin in mice is necessary to estimate whether there are secondary effects. In addition, evaluation of the clinical

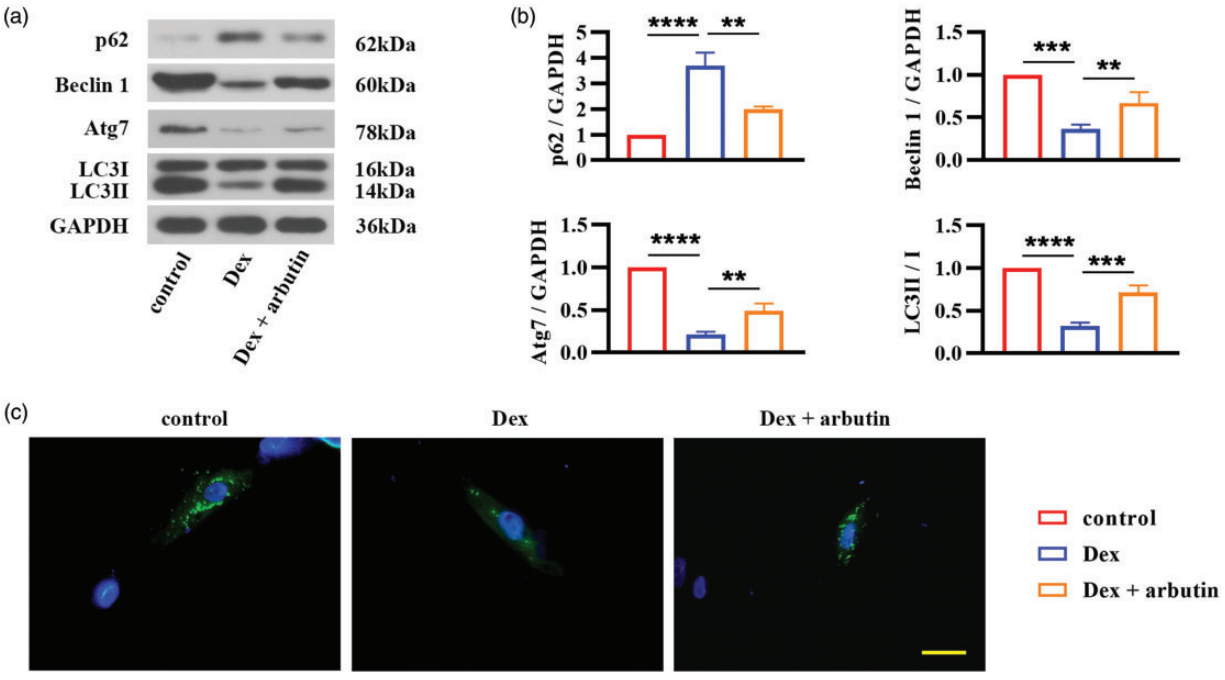

Figure 5. Arbutin activated autophagy in Dex-stimulated MC3T3-E1 cells. (a, b) After treating MC3T3-E1 cells with 1 μM DEX and 100 μM arbutin for three days, the levels of autophagy-related proteins were determined via Western blot analysis, and relative expression was calculated. (c) MC3T3-E1 cells were transfected with pBABEpuro GFP-LC3 plasmid, followed by treatment with DEX and arbutin for three days. LC3 accumulation was observed by GFP puncta. Scale bar, 50 μm. All data are represented as mean ± SD ($n = 3$/group). **P<0. 01, ***P<0.001, ****P<0.0001. (A color version of this figure is available in the online journal.)
GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

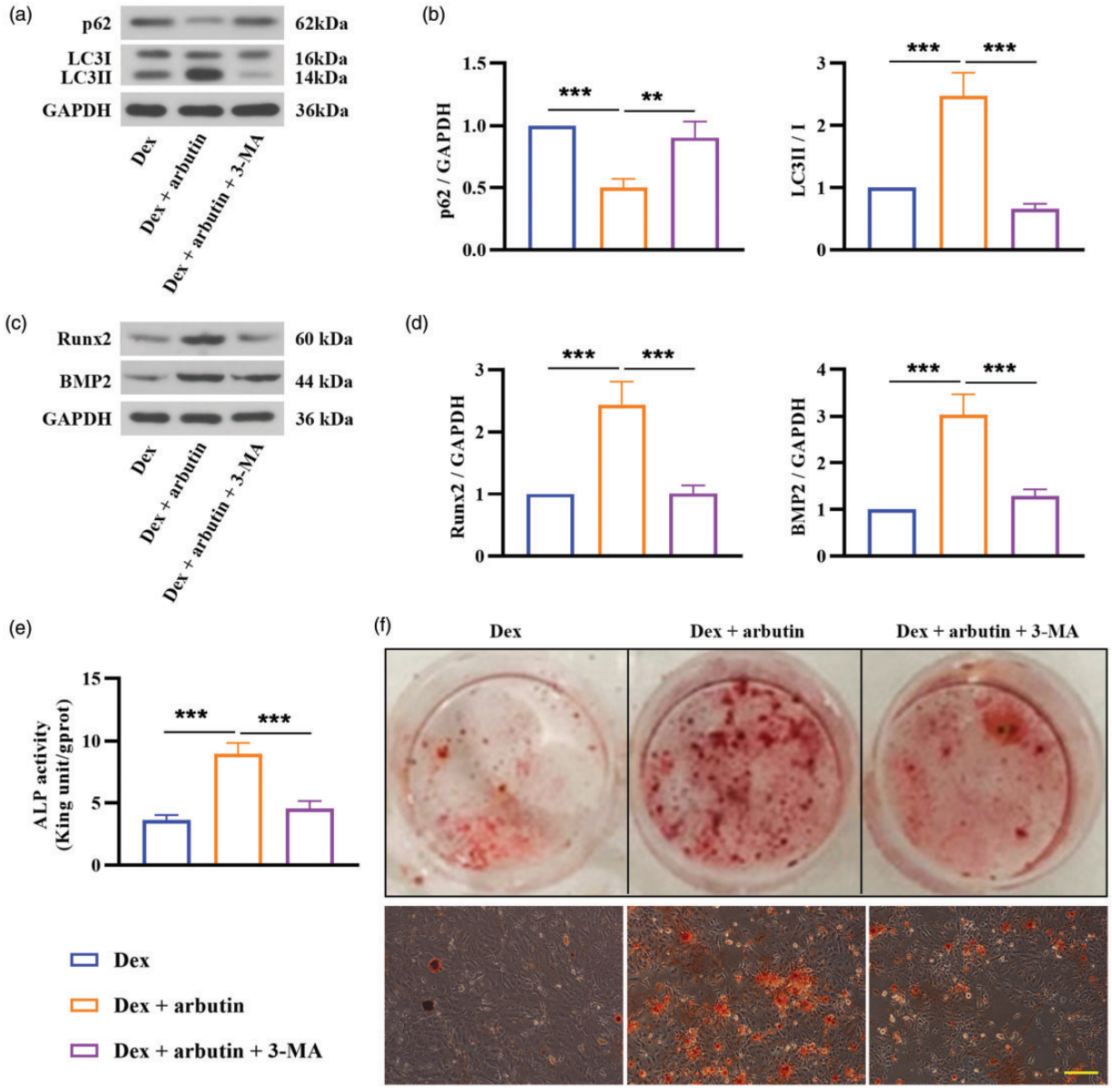

Figure 6. Arbutin facilitated differentiation and mineralization of Dex-treated MC3T3-E1 cells via activating autophagy. MC3T3-E1 cells were cultured in osteogenic medium containing 1 μM DEX and 100 μM arbutin in the presence or absence of 1 mM 3-MA. (a, b) After treatment for three days, the expression of p62, LC3-II, and LC3-I expression was detected, and quantitative analysis was performed. (c, d) The protein level of Runx2 and BMP2 following treatment for seven days. (e) ALP activity was determined on the seventh day. (f) Mineralization degree was detected by Alizarin red staining on the 18th day. Lower panel: matrix mineralization was visualized using a microscope. Scale bar, 200 μm. All data are represented as mean ± SD ($n = 3$/group). **P<0. 01, ***P<0.001. (A color version of this figure is available in the online journal.)
GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ALP: alkaline phosphatase.

efficacy and safety of arbutin in human osteoblasts or osteoporotic patients is also needed in future research.

Glucocorticoid has been shown to trigger imbalanced bone remodeling.[27] Bone remodeling encompasses osteoblast-regulated bone formation and osteoclast-regulated bone resorption, and upsetting the equilibrium of these two processes leads to pathogenesis of osteoporosis.[28,29] All kinds of coupling factors are implicated in these processes and sustain bone remodeling homeostasis. BMP2 is a growth factor that modulates osteoblast differentiation by activating osteoblast-related transcriptional factors, such as Runx2.[30] Runx2 directly binds to the promoter regions of bone matrix protein genes (e.g. ALP, collagen I, osteocalcin, and osteopontin) to regulate their expression.[31,32] ALP is both an early differentiation marker[33] and an enzyme that initializes mineralization.[34] Osteocalcin, a late-stage marker for osteogenesis, is highly expressed during osteoblast mineralization.[35] CTX-1 is a degradation product of collagen I, reflecting the absorption activity of osteoclasts.[36] TRAP is an enzyme that is mainly expressed in osteoclasts and which regulates osteoclast differentiation.[37] It is reported that some drugs regulate the expression of osteoblastic and osteoclastic markers, thereby reversing glucocorticoid-induced osteoporosis. For example, extracts

from plastrum testudinis prevent and manage glucocorticoid-induced osteoporosis by targeting Runx2 and other markers.[38] Treatment of atypical adamantyl retinoid ST1926 increases ALP level and reduces TRAP positive cells, which attenuates bone loss in the femoral head of steroid-induced rats with osteoporosis.[39] To date, there is no research to investigate the role of arbutin in bone formation and bone resorption in glucocorticoid-induced osteoporosis. Our study revealed that arbutin treatment promoted bone formation (reflected by increased BMP2 and Runx2 expression, ALP activity, and osteocalcin level) and inhibited bone resorption (reflected by decreased CTX-1 level and TRAP activity) in Dex-induced osteoporotic mice.

Bone formation largely depends on differentiation and matrix mineralization of osteoblasts. The appearance of ALP activity indicates osteogenic differentiation and mineralization are increased along the process of bone formation.[40] Matrix mineralization is a process that enhances the hardness and strength of bone. In this process, calcium and phosphate crystals produced by bone-forming cells are deposited precisely in the fibrous matrix or scaffold of the bone. Dysregulation of this process causes too little or too much minerals, which leads to weak or brittle bones.[41] Therefore, the effect of arbutin on osteoblast function was investigated via ALP activity and Alizarin red staining. Arbutin treatment enhanced ALP activity in Dex-induced MC3T3-E1 cells, suggesting arbutin increased osteoblast differentiation. Alizarin red dye combines with Ca^{2+} ions, and calcified nodule formation indicates matrix mineralization. Arbutin treatment increased the formation of calcium nodules in Dex-induced MC3T3-E1 cells. This provided *in vitro* evidence that arbutin treatment improved the inhibitory effects of Dex on the differentiation and mineralization of MC3T3-E1 cells.

Autophagy is a catabolic pathway that removes or recycles impaired organelles and dysfunctional proteins, and this has cytoprotective effects on cell growth, survival, proliferation, and differentiation.[40] The process of autophagy exists at a basic level in all cell types and is essential to maintain cell homeostasis and response to stress. There is growing evidence that autophagy plays a crucial role in osteoblast-mediated bone formation. For example, pro-osteogenic marker BMP2 activates autophagy-related factor Atg7, which sequentially regulates Wnt16 signaling to activate MMP-13 and ultimately induces osteoblastic differentiation.[42] AMPK activation and upregulation of autophagy are involved in insulin-like growth factor I (IGF-I)/insulin-like growth factor binding protein 2 (IGFBP-2)-stimulated osteoblast differentiation.[43] Li *et al.*[44] reported that osteoblast-specific conditional deletion of Atg7 leads to decreased osteoblast differentiation and mineralization through triggering ER stress. It is apparent from the evidence described above, that basal autophagy is required in osteoblasts to ensure physiological homeostasis. On the other hand, autophagy is activated and leads to differentiation of osteoclasts during receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclastogenesis.[45,46] A variety of preclinical studies has suggested that inhibition of autophagy reduces osteoclastogenesis and may be a potential strategy for antiosteoporosis treatment. For instance, chloroquine, an autophagy inhibitor, suppresses glucocorticoid-induced osteoclast differentiation and protects mice from bone loss.[47] Mice lacking Beclin 1 (a protein involved in autophagy initiation) in CTSK-expressing cells shows damaged osteoclast function.[45] 3-MA, another autophagy inhibitor, can interrupt osteoclastogenesis stimulated by glucocorticoids.[46] In our study, we found with the progression of Dex-induced osteoporosis, autophagy of osteoblasts was inactivated, which further reduced bone formation and bone mass. Pharmacological induction of autophagy with arbutin rescued the suppressive effects of Dex on osteoblast differentiation and mineralization, thereby alleviating the osteoporotic phenotype in Dex-induced mice. However, the effect of arbutin on autophagy activation in Dex-induced osteoblasts should further be confirmed using electron microscopy, which would provide compelling evidence to our findings. In addition, the current study focused on the effects of arbutin on osteoblast function and autophagy activation, and further research on the role of arbutin in osteoclast function and autophagy will be conducted in our next study.

Collectively, our findings highlight that arbutin ameliorated Dex-induced osteoporosis by facilitating differentiation and mineralization of osteoblasts, which was mediated via autophagy activation. This study provides novel information about the mechanism for the antiosteoporotic activity of arbutin.

AUTHORS' CONTRIBUTIONS

QF contributed to the concept or design of the research. YZ and ML conducted the experiments. ZL analyzed the data. YZ wrote the article. QF revised the article. All authors approved the final version of the article for publication.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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