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Prevention of glucocorticoid induced bone changes with beta-ecdysone

Weiwei Dai^{1,2}, Li Jiang¹, Yu-An Evan Lay¹, Haiyan Chen¹, Guoqin Jin², Hongliang Zhang¹, Alex Kot¹, Robert O. Ritchie³, Nancy E. Lane¹, and Wei Yao¹

¹Center for Musculoskeletal Health, Internal Medicine, University of California at Davis Medical Center, Sacramento, CA 95817 USA

²Department of Science and Technology, Longhua Hospital Shanghai University of Traditional Chinese Medicine, Shanghai, China, 200032

³Department of Materials Science and Engineering, University of California at Berkeley, Berkeley, CA 94720 USA

Abstract

Beta-ecdysone (β Ecd) is a phytoecdysteroid found in the dry roots and seeds of the asteraceae and achyranthes plants, and is reported to increase osteogenesis *in vitro*. Since glucocorticoid (GCs) excess is associated with a decrease in bone formation, the purpose of this study was to determine if treatment with β Ecd could prevent GC-induced osteoporosis. Two-month-old male Swiss-Webster mice (n=8-10/group) were randomized to either placebo or slow release prednisolone pellets (3.3mg/kg/d) and treated with vehicle control or β Ecd (0.5mg/kg/d) for 21 days. GC treatment inhibited age-dependent trabecular gain and cortical bone expansion and this was accompanied by a 30-50% lower bone formation rate (BFR) at both the endosteal and periosteal surfaces. Mice treated with only β Ecd significantly increased bone formation on endosteal and periosteal bone surfaces, and increased cortical bone mass were their controls to compare to GC alone. Concurrent treatment of β Ecd and GC completely prevented the GC-induced reduction in BFR, trabecular bone volume and partially prevented cortical bone loss. *In vitro* studies determined that β Ecd prevented the GC increase in autophagy of the bone marrow stromal cells as well as in whole bone. In summary, β Ecd prevented GC induced changes in bone formation, bone cell viability and bone mass. Additional studies are warranted of β Ecd for the treatment of GC induced bone loss.

Keywords

Beta-ecdysone (β Ecd); glucocorticoid; bone formation; autophagy

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Corresponding Author: Wei Yao, MD, Department of Medicine, Center for Musculoskeletal Health, University of California at Davis Medical Center, Sacramento, CA 95817, Telephone: 916-734-0763, Fax: 916-734-4773, wei.yao@ucdmc.ucdavis.edu.

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Introduction

Glucocorticoids (GCs) are frequently used in clinical medicine to treat non-infectious inflammatory diseases. However, GC use results in rapid trabecular bone loss and a high incident fracture risk [1, 2]. Lower peak bone mass acquisition, presence of osteopenia and vertebral collapse were often observed in children with primary increase in the endogenous levels of GCs with Cushing's disease [3, 4] or on GC treatments for some chronic diseases such as asthma [5] and other inflammatory diseases [6, 7]. Children treated with chronic GCs normally have growth retardation including the suppression of bone growth [8, 9]. Prevention for and treatment of glucocorticoid-induced osteoporosis (GIOP) in adults include bisphosphonates (BPs) and PTH [10-13]. The former have also been used to treat children with GIOP [14, 15]. However, as bone is highly remodeled during childhood to maintain adequate mineralization of the rapidly growing skeleton, the use of BPs is not ideal as they inhibit bone remodeling and could increase the mineral in the bone matrix, which may not be ideal to use in a growing skeleton [16, 17]. To this end, continued and safety studies for the use of BPs in children have yet to be established [18-20].

Recently, naturally-derived products contain a variety of molecules with potent biological activities. Phytoecdysteroids are plant-derived ecdysteroids that are structural analogs of insect molting hormone ecdysone, which are critical for insects to maintain “eat-to-reproduce” life cycle [21]. Beta-ecdysone (β Ecd) is one of the most abundant phytoecdysteroids found in plants, such as in the dry roots and seeds of the asteraceae and achyranthes, as well as in spinach, quinoa and suma root [22, 23]. These plants are often used in the traditional Chinese medicine to help to reduce joint and back pain. It has been shown that β Ecd increases protein synthesis and reduces protein degradation in the skeletal muscle cells [24, 25]. As it increases muscle weight in rodents [26-28], β Ecd has been referred to as an “anabolic” naturally-derived supplement [29]. Additionally, β Ecd is also found to stimulate mesenchymal stem cells' osteogenic differentiation but to inhibit their adipogenic differentiation [30]. β Ecd is reported to increase the growth plate width in estrogen deficient rats and to have a marginal beneficial effect on trabecular bone and cartilage preserving following ovariectomy (OVX) [31, 32]. Since GC use in children often results in growth retardation, [6, 7, 33] through GC induced inhibition of osteoblasts through multiple mechanisms [34], we seek to determine if β Ecd can rescue the GC-suppression on bone formation. We have hypothesized that β Ecd treatment inhibits bone loss and deterioration of mechanical properties associated with GC uses, partially through maintenance of bone formation. Also, we explore osteoblast and osteocyte autophagy following GC or with concurrent β Ecd treatment, and evaluate if autophagy is one of the mechanisms explaining the bone anabolic effect we observed for β Ecd.

Methods

Animals and Experimental Procedures

Two-month-old male Swiss-Webster mice were maintained on commercial rodent chow (22/5 Rodent Diet; Teklad, Madison, WI) available *ad libitum* with 0.95% calcium and 0.67% phosphate. Mice were housed in a room that was maintained at 20 °C with a 12-hour light/dark cycle. They were randomized into 4 experimental groups of 8 animals in each

group. Slow release pellets (Innovative Research of American, Sarasota, FL) of prednisolone (GC) were implanted respectively: Group 1, the control group, was implanted with a placebo pellet (PL); Group 2 was implanted with PL pellet + β Ecd (PL + β Ecd 0.5mg/kg, 5 \times /wk); Group 3 was implanted with a prednisolone 5mg/60 day slow-release pellet, which is equivalent to 3.3 mg/kg/d (GC), and Group 4 was implanted with prednisolone 5mg/60 days slow-release pellet + β Ecd (GC + β Ecd 0.5mg/kg, 5 \times /wk). The mice were sacrificed after three weeks of treatments. The β Ecd dose was based publications on myogenesis and our *in vitro* experiments on osteogenesis and osteoclastogenesis using β Ecd doses ranging from 10^{-3} to 10^{-9} M [25, 26].

β Ecd was purchased from Sigma-Aldrich (St. Louis, MO). Calcein (30mg/kg) was injected to all mice seven and two days before euthanization. All animals were treated according to the USDA animal care guidelines with the approval of the UC Davis Committee on Animal Research.

Measurements of serum hormonal levels and biochemical markers of Bone Turnover

The mice were fasted overnight before their serums were collected for the measurements of cortisol, leptin and insulin using a luminex multiplexing hormonal panel assay while bone turnover markers, osteocalcin and osteoprotegerin (OPG) levels were measured using a luminex multiplexing bone panel assay (EMD Millipore, Billerica, MA, USA). Serum CTX-1 was measured by ELISA (Immunodiagnostic Systems Inc., Gaithersburg, MD, USA).

Assessment of bone mass and bone microarchitecture

The 5th lumbar vertebral body and the right femur mid-diaphysis from each animal were scanned and measured by MicroCT (VivaCT 40, Scanco Medical, Bassersdorf, Switzerland), with an isotropic resolution of 10.5 μ m. Bone samples were scanned at 70 kVp and 145 μ A. Three-dimensional trabecular structural parameters were measured directly, as previously described [35]. *Ex vivo* microCT scans of the central right femur that included a region of total 100 slices. All the slices were used to evaluate total volume (TV), cortical bone volume (BV), and cortical thickness (Ct.Th) [36-39].

Assessment of surface-based bone turnover by bone histomorphometry

The third and fourth lumbar vertebral bodies (LVB) were fixed in 4% paraformaldehyde for 24 hours, and then soaked in 30% sucrose in PBS at 4°C for eight hours and then embedded in optimum cutting temperature compound. Eight μ m thick frozen sections were obtained using a Leica microtome coupled with a CyroJane tape transfer system. The slides were mounted using 50% glycerol in PBS. Bone histomorphometry was performed using a semi-automatic image analysis Bioquant system (Bioquant Image Analysis Corporation, Nashville, TN) [35]. Static measurements included total tissue area (T.Ar), bone area (B.Ar) and bone perimeter (B.Pm). Dynamic measurements included single- (sL.Pm) and double-labeled perimeter (dL.Pm), and interlabel width (Ir.L.Wi). These indices were used to calculate 2-D bone volume (B.Ar/T.Ar), trabecular number (Tb.N), trabecular thickness (Tb.Th), and mineralizing surface (MS/BS and mineral apposition rate (MAR). Surface-based bone formation rate (BFR/BS) was calculated by multiplying mineralizing surface

(single labeled surface/2 + double labeled surface) by MAR [40]. A separated section was used to stain for tartrate-resistant acid phosphatase (TRAP) to measure osteoclast number at the trabecular bone surface (OC/BS). We used the terminologies following the recommendation of the American Society for Bone and Mineral Research and we have reported similar methodology in other experiments in our laboratory [36, 41].

The femoral shafts were dissected and fixed in 4% paraformaldehyde, dehydrated in graded concentrations of ethanol and xylene, embedded un-decalcified in methyl methacrylate and then cross-sectioned using a SP1600 microtome (Leica, Buffalo Grove, IL, USA) into 40 μ m sections. Total cross-sectional bone area (T.Ar), cortical area (Ct.Ar), and cortical thickness (Ct.Th) were measured with the Bioquant Image analysis system. Single and double labeled surface, and inter-labeled width were measured separately at the endocortical (Ec.) and periosteal (Ps.) bone surfaces. MAR and BFR/BS were calculated thereafter for both the endocortical and periosteal bone surfaces [36-39].

Biomechanical testing

For the vertebrae, the endplates of the lumbar vertebral body were polished using an 800-grit silicon carbide paper to create two parallel planar surfaces. Before testing, caudal and cranial diameter measurements were taken at the top, middle, and bottom of LVB6 to obtain six measurements which were averaged as the diameter; the height along the long axis was recorded as well and the vertebrae were modeled as a cylinder. Each lumbar vertebra was then loaded to failure under unconfined compression along its long axis using an MTS 831 electro-servo-hydraulic testing system (MTS Systems Corp., Eden Prairie, MN) at a displacement rate of 0.01 mm/s with 1 kN load cell; the tests were performed in 37°C HBSS and sample loads and displacements were continuously recorded throughout each test. Values for the maximum load and maximum stress (bone strength) for compression were then determined, where the stress was calculated using $\sigma=4P/(\pi d^2)$, with P being the load and d the average diameter.

To analyze the biomechanical properties of the femurs, the femoral samples were subjected to three-point bending tests, with the bone loaded using an MTS 831 electro-servo-hydraulic testing system (MTS Systems Corp., Eden Prairie, MN, USA) such that the posterior surface was in tension and the anterior surface was in compression. The major loading span was 14.5 mm. Each femur was loaded to failure in 37°C HBSS at a displacement rate of 0.01 mm/s while its corresponding load and displacement were measured using a calibrated 1 kN load cell. Two diameter measurements were taken at the fracture location, and averaged to model the femur as a cylinder. Values for the maximum load and ultimate strength of bending tests were then determined, with the stress calculated from $\sigma = PLy/4I$, where P is the load, L is the major loading span, y is the distance from the center of mass ($d/2$), and I is the moment of inertia ($\pi d^4/64$), with d being the average diameter. A measure of toughness was estimated in terms of the work of fracture, specifically the area under the load vs/ displacement curve normalized by twice the fracture surface area [36, 42].

***In vitro* osteogenesis and adipogenesis assays**

Bone marrow stromal cells (BMSC) were flushed out from long bones obtained from the 2-month-old male mice. For adipogenesis differentiation, the BMSCs were cultured using a STENPRO Adipogenesis Differentiation Kit (GIBCO Invitrogen Cell Culture) for 10 days and stained with Oil Red O for lipid deposits. RNA was extracted from day 14 cultures for quantitative measurements of RNA levels for genes associated with osteogenesis (*Runx2*, *Bglap1*) or adipogenesis (*Cebp-a* or *Ppar-γ*). For osteogenic differentiation, BMSCs were cultured for 14 days in osteogenic media and then the colony-forming unit-forming colonies (CFU-F) were stained by crystal violet followed by alizarin red staining for osteogenic colonies (CFU-Ob) (Sigma-Aldrich, St. Louis, MO, USA) [37]. Subsequently, stained cells were eluted from membranes and absorbance was measured at 590 nm (crystal violet) or 410 nm (ALP) [38, 43]. In a separated experiment, BMSC cells were obtained from male dsRed-LC3 reporter mice of Swiss Webster background (made by UC Davis Mutant Mouse Regional Resource Center; property of Drs. Yao and Lane). The BMSC were cultured in osteogenic medium for seven days before they were treated with PBS, Dexamethasone (Dex, 10^{-6} M), β Ecd (10^{-7} M) or combination of Dex + β Ecd in serum-starved conditions for eight hours. The cells were either lysed to collect protein or fixed with 4% paraformaldehyde, and examined under a Keyence Imaging System with cell count software (Keyence Corp. of America, Itasca, IL, USA). Autophagic cells were quantified by counting cells exhibiting 10 or more dsRed-LC3 dot/cells.

Real-time RT-PCR

Total RNA was obtained from the distal tibiae. Total RNA was isolated using a modified two-step purification protocol employing homogenization (PRO250 Homogenizer, 10mm \times 105mm generator, PRO Scientific IN, Oxford CT) in Trizol (Invitrogen, Carlsbad, CA, USA). The autophagic focus RT-PCR gene pathway arrays and the primer sets were purchased from SABioscience, a Qiagen company, (Frederick, MD, USA). Each pathway gene array has pre-selected 96 genes that are related to autophagy pathways, housekeeping genes, and no primer or cDNA controls. Detailed gene information can be found at <http://www.sabiosciences.com/RTPCR.php>. We excluded genes with Ct values of > 35 for the pathway analysis [34, 44].

Western Blot

Tibial cortical bones were lysed in RIPA buffer with homogenization. The bone lysates were resolved on SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies that include β -actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Atg 7, anti-Atg-16L and anti-LC3 (Cell Signaling Technology, Danvers, MA, USA) followed by species-specific horseradish peroxidase secondary antibody. Anti-LC3 antibody recognizes both LC3-I, which is cytoplasmic, and LC3-II that binds to the autophagic membranes. Immunoreactive materials were detected by chemiluminescence (Pierce Laboratories, Thermo Fisher Scientific, Rockford, IL, USA), then were imaged and quantitated by BIO-RAD ChemiDoc MP imaging system and analysis software [36].

Statistical analysis

The group means and standard deviations (SDs) were calculated for all outcome variables. The nonparametric *Kruskal-Wallis* test was used to determine the overall and group-wise differences between the groups. (SPSS Version 14; SPSS Inc., Chicago, IL).

Results

Body weight

Body mass increased by approximately 25% in the PL and PL + β Ecd groups and declined in the GC and GC+ β Ecd groups by 20% during this three-week study period ($p < 0.05$ vs. baseline). β Ecd treatment did not induce any change in body mass as compared to β Ecd naïve groups.

β Ecd treatment alone did not alter serum cortisol, ACTH, Leptin or Insulin levels (Table1) or the estrogen, progesterone, T3, T4 levels (data on file). Serum osteocalcin and CTX-1 levels increased significantly in PL + β Ecd group as compared to PL group. GC did not significantly change serum hormonal levels but decreased serum osteocalcin by 12% and increased serum CTX-1 concentration by 125% ($P < 0.05$ vs. PL). GC + β Ecd tended to increase osteocalcin level and reduced CTX-1 level ($P < 0.05$ vs. GC) as compared to GC group (Table1).

Bone volume and bone turnover changes in the trabecular bones

In PL + β Ecd treatment group compared to the PL, trabecular bone volume/tissue volume (BV/TV) was increased by 41% at the 5th lumbar vertebral body (LVB). On the other hand, GC reduced BV/TV by 32% as compared to the PL group ($P < 0.05$ vs. PL). In GC+ β Ecd compared to the GC, BV/TV was increased by 43% ($P < 0.05$ vs. GC). Trabecular thickness showed similar changes as BV/TV (Figures 1A and B). In the distal femur (DFM), the trends of changes were similar to the LVB but to a lesser degree following GC or with β Ecd treatments. In PL + β Ecd treatment group compared to the PL, trabecular bone BV/TV and Tb.Th were increased by 73% and 47% ($P < 0.05$ vs. PL), respectively, at GCs non-significantly lowered BV/TV and Tb.Th in the DFM and GC+ β Ecd had approximately 10% higher BV/TV and Th.N, but these changes were not significant compared to the GC group (Figure 1C).

More intriguing was that all parameters for trabecular bone formation measured at the 4th LVB, namely bone mineralizing surface (MS/BS), and bone formation rate/BS. In PL + β Ecd treatment group compared to the PL, MS/BS and BFR/BS were increased by 43% and 30% ($P < 0.05$ vs. PL), respectively. In GC treatment group compared to the PL, GC significantly reduced MS/BS by 46%, MAR by 23% and BFR/BS by 60% ($P < 0.05$ vs. PL) while concurrent treatment of β Ecd prevented these inhibitions of GC on bone formation parameters (Figure 2 A and B). In GC treatment group compared to the PL, GC significantly increased the osteoclast number/BS whereas concurrent treatment of β Ecd prevented this change (Figure 2A).

Bone volume and bone turnover changes in the cortical bone

We next measured cortical bone architectural changes at the mid-femoral diaphyses by microCT and bone histomorphometry. When compared to the PL, cortical bone volume (Ct-BV) was increased by 4.5% in PL+ β Ecd ($P < 0.05$ vs. PL), and was reduced by 4% in GC group ($P < 0.05$ vs. PL). β Ecd treatment did not prevent the loss in Ct-BV (Figure 3A). When compared to the PL, bone formation at the endocortical bone surface was increased by 213% in PL+ β Ecd ($P < 0.05$ vs. PL), but reduced by 99% in GC group ($P < 0.05$ vs. PL), and was increased by 93% in the GC+ β Ecd group as compared to GC group ($P < 0.05$ vs. GC). When compared to the PL, bone formation at the periosteal bone surface was increased by 55% in PL+ β Ecd but reduced by 120% in GC group; it was 59% higher than GC in GC+ β Ecd group ($P < 0.05$ vs. GC) (Figures 3 A and B).

Bone strength measurements

The vertebral compression strength was measured by compression test. Compared to PL group, β Ecd treatments resulted in a higher maximum load, ultimate stress and toughness by 53%, 21%, and 16% respectively ($P < 0.05$ vs. PL) (Table 2). On the other hand, GC reduced these parameters by 24%, 19%, and 16% respectively, as compared with the same PL mice ($P < 0.05$ vs. PL). By contrast, GC+ β Ecd mice had higher maximum load, ultimate stress and toughness by 44%, 31%, and 56%, respectively, as compared to GC mice ($P < 0.05$ vs. GC). Likewise, β Ecd induced higher maximum load, ultimate stress and toughness of the femurs by 23%, 42%, and 9% respectively, as compared to PL ($P < 0.05$ vs. PL). Interestingly, in GC-treated mice, there were no significant differences in the maximum load and ultimate stress but the cortical toughness was reduced by 24% as compared to PL ($P < 0.05$ vs. PL). GC+ β Ecd mice had higher maximum load, ultimate stress and toughness by 16%, 18%, and 21%, respectively, as compared to GC mice ($P < 0.05$ vs. GC), which were similar to the PL levels.

Effect of β Ecd on osteogenesis and autophagy

To explore the mechanism of β Ecd on bone formation, we first treated bone marrow stromal cells (BMSCs) with β Ecd (10^{-4} - 10^{-9} M; data from 10^{-5} and 10^{-8} M was presented) in adipogenic or osteogenic media, we found the expression of pro-adipogenic genes (*Cebp- α* and *Ppar- γ*) were lowered by 5-20 fold ($P < 0.05$ vs. control), accompanied by less lipid formation (Figure 4A). On the other hand, BMSCs osteogenic differentiation was stimulated by β Ecd, as shown by an increase in both osteoblastic gene expressions (*Runx2* and *Bglap1*) ($P < 0.05$ vs. control) and ratio of the CFU-Ob to CFU-F (Figure 4B).

To further explore how osteoblast and osteocyte viability was affected by treatment with β Ecd, we evaluated osteoblast autophagy both *in vitro* and *in vivo*. The BMSCs were cultured in osteogenic media for 7 days before they were exposed to Dex or β Ecd. We found the ratio of LC3+ primary osteoblast numbers were decreased by more than 50% in Dex – treated osteoblasts and combination treatment of β Ecd prevented this decrease (Figure 5A and B).

To evaluate the contribution of GC or with the combinational treatment of β Ecd on autophagy *in vivo*, we first extracted RNA from the tibial cortical bone and performed RT-

PCR array on autophagy assay that contained 80 genes associated with autophagy. We found that GC reduced the autophagic gene expression including key genes associated with autophagy induction, such as *Atg7* and *Beclin 1* [45], by 1-5 folds while co-treatment of β Ecd activated these autophagic gene expressions by 5-10 folds (Figure 6A). GC reduced expression of *Atg7* and *Atg16L* by more than 50%, indicating reduced formation of autophagosome. On the other hand, GC decreased LC3-II/I ratio by about 15%, suggesting LC3 conversion might be not significantly affected following GC treatment *in vivo*. Concurrent of GC and β Ecd treatment maintained protein levels of *Atg7*, *Atg16L* and the ratio of LC3II/I to PL control level (Figure 6B and C).

Discussion

In this report, we used two-month-old Swiss-Webster male mice and treated them with slow release prednisolone pellets to study the negative effect of GCs on bone growth and to see if treatment with the β Ecd and GCs would alter bone growth. We found that three weeks of β Ecd treatment alone or with combination of GC treatment altered the gain in body mass. β Ecd treatment alone increased bone formation primary by increasing the osteoblast numbers (mineralizing surface) such that surface-based bone formation rate was significantly increased. The substantial improvements in bone formation in β Ecd-treated mice translated into substantially higher bone mass with higher vertebral and cortical bone strength. Detrimental effect on both trabecular and cortical bone architecture and bone strength were apparent in mice receiving GCs, which included inhibitions in active bone formation at the trabecular bone and cortical bone expansion. β Ecd treatment partially precluded inhibition of bone formation induced by GC, especially at the lumbar vertebrae and at the periosteal surface of the femoral shafts. Furthermore, our results demonstrated that β Ecd increased the differentiation of mesenchymal progenitor cells towards osteoblast *in vitro*, consistent with the observation of increasing the osteoblast surface following β Ecd treatment *in vivo*.

As GCs potently suppress osteoblast activities and adversely affect bone mineralization [41, 46], we hypothesized that therapies that target bone-forming capability would be superior to anti-resorptive agents. In support of this notion, it is reported that anabolic agent, hPTH (1-34), is superior to bisphosphonates in increasing spine and hip BMD and reduces the incidence of new vertebral fractures in glucocorticoid-induced osteoporosis (GIOP) populations for both men and women [11, 47-49]. In clinical practice, both bisphosphonates and PTH increase the spine BMD to a greater extent than the hip BMD [12, 14, 47, 50-53]. Prolonged bisphosphonates treatment may have an adverse effect on cortical bone mineralization and quality such that cortical strength decline independent of bone mass [54]. Prolonged GC treatments further worsen the bone quality [35, 55], and atypical fractures were more common in GIOP patients receiving bisphosphonate treatments ([56]). The effect of hPTH (1-34) on hip fracture reduction has not been shown in clinical studies in GIOP populations. [55, 57]. In our current study, we found GCs reduced vertebral bone strength, an observation that is consistent with bone loss [35, 41]. Interestingly, cortical bone maximum load and maximum stress measurements for cortical bone remained unchanged despite a decrease in bone volume. However, GC significantly reduced toughness, a measure of overall bone quality that measures the resistance to fracture [58]. This finding again

suggests the adverse effect of GC on the cortical bone quality may not correlate with whole bone mass or bone mineral density [35, 59]. On the other hand, β Ecd completely prevented losses in mechanical (eg. Maximum load) and material strengths (stress and toughness) in both the vertebral trabecular bone and femoral cortical bone despite it did not completely prevent GC-induced bone loss in these two bone sites. These findings suggest β Ecd could be a good alternative treatment for GC-induced bone fragility.

β Ecd treatment had an inconsistent effect on bone resorption in that it inhibited osteoclasts maturation *in vitro* (data on file) while increasing serum CTX-1. However, serum was collected only at one single time point (eg, at the end of three months) and thus the serum measurements may not reflect the dynamic changes over the entire experiment on osteoclast activities. Similar to PTH, β Ecd treatment may activate bone turnover, increasing both bone resorption and bone formation, with the latter exceeding the former such that a net bone gain was observed [60].

Since GC reduced the body weight of the study mice by more than 20%, it was possible that the reduced body weight might have contributed to the GC-induced bone loss as well as the reduction in treatment efficacy for β Ecd as GC dose was increased. We do not think this was the case as we evaluated the changes between weight-bearing bone site (distal femurs) and the non-weight bearing bone site (lumbar vertebral bodies) and found similar trends for GC or GC + β Ecd with regards to bone formation and bone mass.

Mechanistically, β Ecd is reported to promote muscle cell growth via insulin and PI3K/Akt signaling and is reported to inhibit NF- κ B activation in a cancer cell line [21, 25, 27, 61]. β Ecd binds to ecdysone receptor with EC50 of 0.3nm, a ligand-activated transcriptional factor found in arthropods [62, 63]. Since β Ecd is structurally similar to testosterone, it is considered to have a steroid hormone-like effect. However, β Ecd is shown to have no direct binding to the androgen receptor or other cytosolic steroid receptors [25, 64, 65], suggesting that our observation of an anabolic effect of β Ecd on male skeleton may be independent of its androgen-like structure. Moreover, we did not observe that β Ecd altered serum hormone levels of cortisol, estrogen, progesterone, T3, T4, insulin and leptin, suggesting that β Ecd had a direct effect on the bone metabolism. Our finding that β Ecd stimulated bone marrow stromal cells differentiating into osteoblasts supports a direct effect on bone cells.

Although far from conclusive, our exploratory experiments on autophagy *in vitro* and *in vivo* supported that β Ecd might sustain the autophagic level in osteoblastic-like cells and in bone, whereas GC suppressed the induction of autophagy. Endogenous GCs are the main hormones released in response to stress and GC excess has been associated with nutrition deprived and accelerating aging process [66, 67]. We have reported that osteocyte autophagy is crucial to the regulation of bone structure and plays a role in GC induced bone fragility [44, 68]. It was shown that the removal of Atg7, a gene essential for autophagy initiation, resulted in low bone mass [69]. Autophagy is not a simple on – off phenomenon allowing a quick judgment of good/protective or bad/detrimental effect on cell metabolism. Rather, the level of autophagy has been maintained at a fine equilibrium so if or when the stress-inducing agent is removed, the cells might go on to survive. However, a sustained state of cellular stress, such as with high dose or chronic GC treatments, could result in

failure of the induction of autophagy. Based on our results we propose that osteoblasts and osteocytes initially respond to GC induced stress by increasing the number of cells that undergo autophagy. The stress from low dose GC is nontoxic, allowing the cells to respond to the initial cellular or nutritional insults, which become no longer effective when the GC dose is high or with prolonged treatment. Defective autophagy may play a crucial role in maintaining bone integrity, especially in GC-induced bone fragility. The maintenance of bone cell viability through autophagy might partially explain β Ecd effect on osteogenesis and on bone formation. β Ecd may represent an important therapeutic option in the prevention of GC induced bone fragility by sustaining the level of autophagy in bone cells.

This study has several limitations. First, only male mice were studied one time point (21 days), with a single dose regimen of β Ecd. Other studies will need to be done to determine if our result is present in female mice and if there is a dose response to the treatment. Second, this study only addressed the effect of β Ecd for the prevention of GC induced bone loss. To evaluate if β Ecd can increase bone mass in mice treated chronically with GCs will require another study. We used relatedly young mice that we would have to carefully dissect out the effect of GC and β Ecd on bone growth inhibition verse a true decrease in bone volume. We did not measure the length of long bones and could not conclude if GC or β Ecd affect growth. It has been shown that GCs normally decrease linear bone growth in animals and decrease bone mass, which are different from young patients on GCs who usually gain weight. Glucocorticoid-induced bone changes in children are usually confounded by the underlying disease and the changes in body mass [6, 7, 33]. The mice we used in the study gained about 20% body mass during the study, which might be similar to the skeleton of early adulthood finishing its maturation. Compared to the other studies that we and others have published on GIOP that used skeletal matured mice, we found GC excess in the young growing have more profound inhibition on periosteal expansion with similar degree of trabecular bone loss [35, 41]. Nevertheless, we showed that changes in bone volume correspond to changes in bone strength with GC or following β Ecd treatment. These observations were similar between loaded or unloaded skeletal sides suggesting our findings were independent of body mass.

In summary, we have found that short-term administration of β Ecd in growing male mice resulted in marked enhancements in both trabecular and cortical bone formation that were associated with significantly increased trabecular and cortical bone volume, both contributing to overall skeletal strength. These findings implicate the potential use of β Ecd in augmenting peak bone mass. While GC treatment reduced endosteal bone formation and periosteal bone expansion, β Ecd treatment prevented the detrimental effect of GC on bone formation, especially on trabecular bone. These results provide a strong pre-clinical support for testing the ability of β Ecd treatment to improve skeletal fragility resulting from GC excess.

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Highlights

1. Beta-ecdysone (β Ecd) completely prevented the GC-induced reduction in trabecular bone formation and bone strength.
2. β Ecd partially prevented the GC-induced reduction in cortical bone formation and bone strength.
3. β Ecd stimulated bone marrow stromal cells differentiation into osteoblast
4. β Ecd maintained the autophagy level in bone, which was otherwise suppressed by GC.

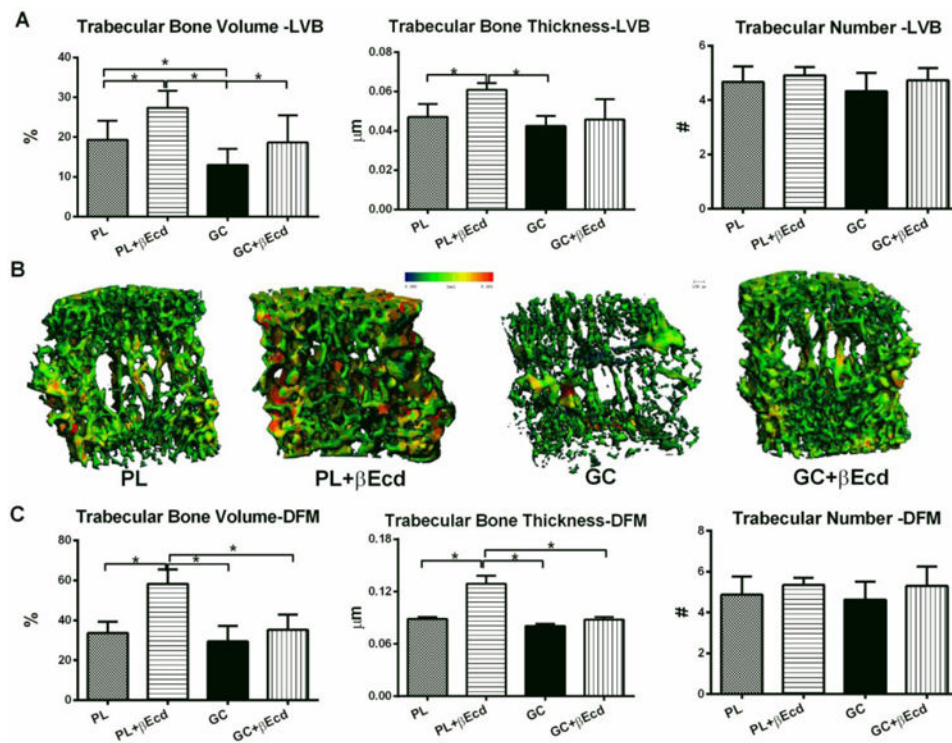


Figure 1. Effects of GC and β Ecd treatments on the vertebral and femoral trabecular bone microarchitectures, assessed by microCT. Two-month-old mice were treated with β Ecd, GC or concurrent treatment of GC + β Ecd for 21 days. (A) Lumbar vertebral trabecular bone (LVB) structure measured by micro-CT. (B) Representative trabecular thickness maps were obtained from the LVB by micro-CT where the trabecular thickness is color coded: with blue-green colors indicate thinner trabeculae whereas yellow-red colors are used for thicker trabeculae. (C) Distal femoral trabecular bone (DFM) structure as measured by microCT. *: $p < 0.05$ between indicated groups.

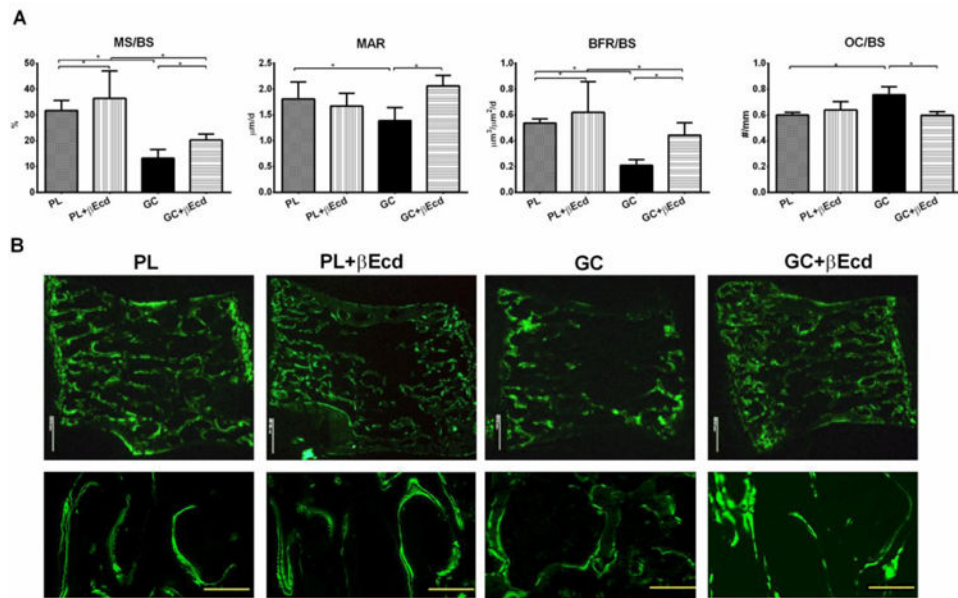


Figure 2. Effects of β Ecd on trabecular bone formation. (A) Surface-based bone formation was measured at the un-decalcified LVB frozen sections. (B) Representative LVB sections from the treatment groups. *: $p < 0.05$ between indicated groups.

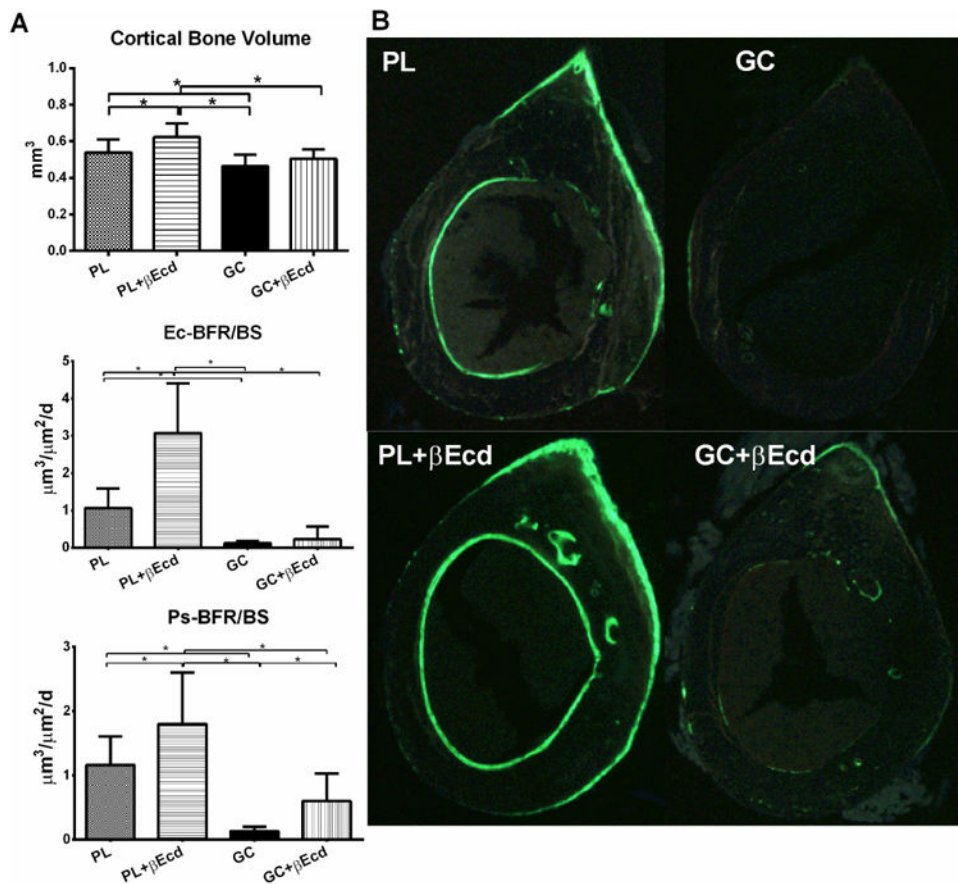


Figure 3. Effect of GC and βEcd treatments on cortical bone structure and surface-based bone turnover, assessed by microCT and bone histomorphometry. (A) Cortical bone volume and bone formation were measured at the mid-shaft of the femur. (B) Representative cross-sectional cortical bone sections from the mid-shaft of the femurs. *: $p < 0.05$ between indicated treatment groups.

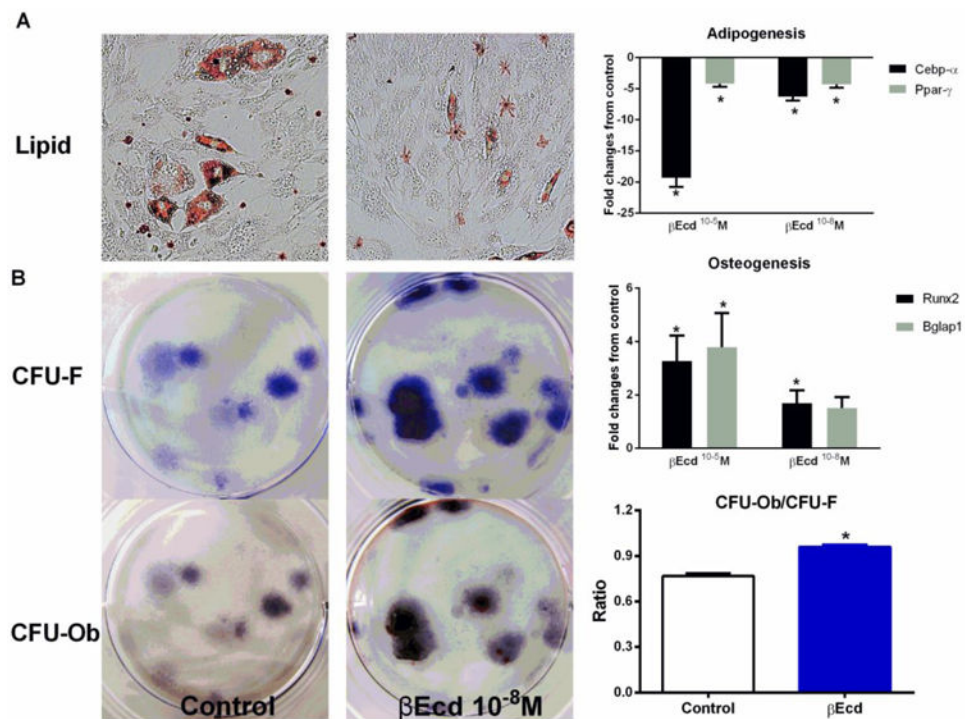


Figure 4. β Ecd increases osteogenic differentiation of bone marrow stromal cells. Bone marrow cells were collected from long bones of male mice, two months of age and maintained in α -MEM with 10% FBS and antibiotics for four days. The adherent cells were collected and cultured in adipogenic or osteogenic media for 21 days. (A) Adipogenic differentiation measured by oil red staining and gene expressions related to adipogenesis (Cebp- α and Ppar- γ). (B) Osteogenesis measured the ratio of CFU-Ob/CFU-F and genes associated with osteoblast differentiation (Runx2 and Bglap1). Data are means \pm SD. *: $p < 0.05$ vs. control (PBS). All the studies were performed in triplicate.

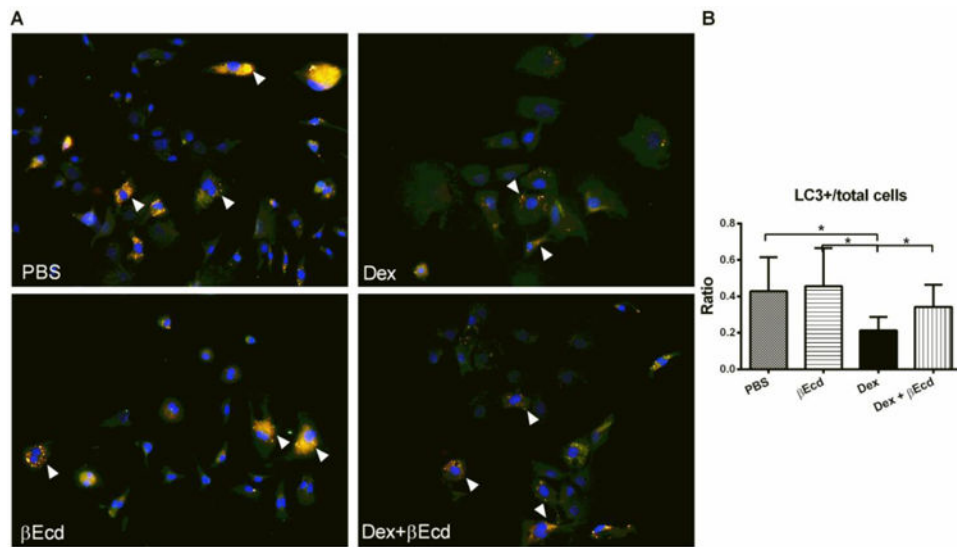


Figure 5. Effects of β Ecd on osteoblast autophagy *in vitro*. BMSC cells were obtained from dsRed-LC3 reporter male mice and cultured in osteogenic medium for 10 days before they were treated with PBS, Dexamethasone (Dex, 10^{-6} M), β Ecd (10^{-7} M) or combination of Dex + β Ecd in serum-starved conditions for eight hours. (A and B) Representative images (A) and quantitation of (B) dsRed-LC dots (autophagosomes, white arrows) in BMSC grown in osteogenic medium for 7 days and after 8 hours serum starvation incubation with Dex or β Ecd as indicated. More than 200 cells were analyzed per sample. All the studies were performed in triplicate.

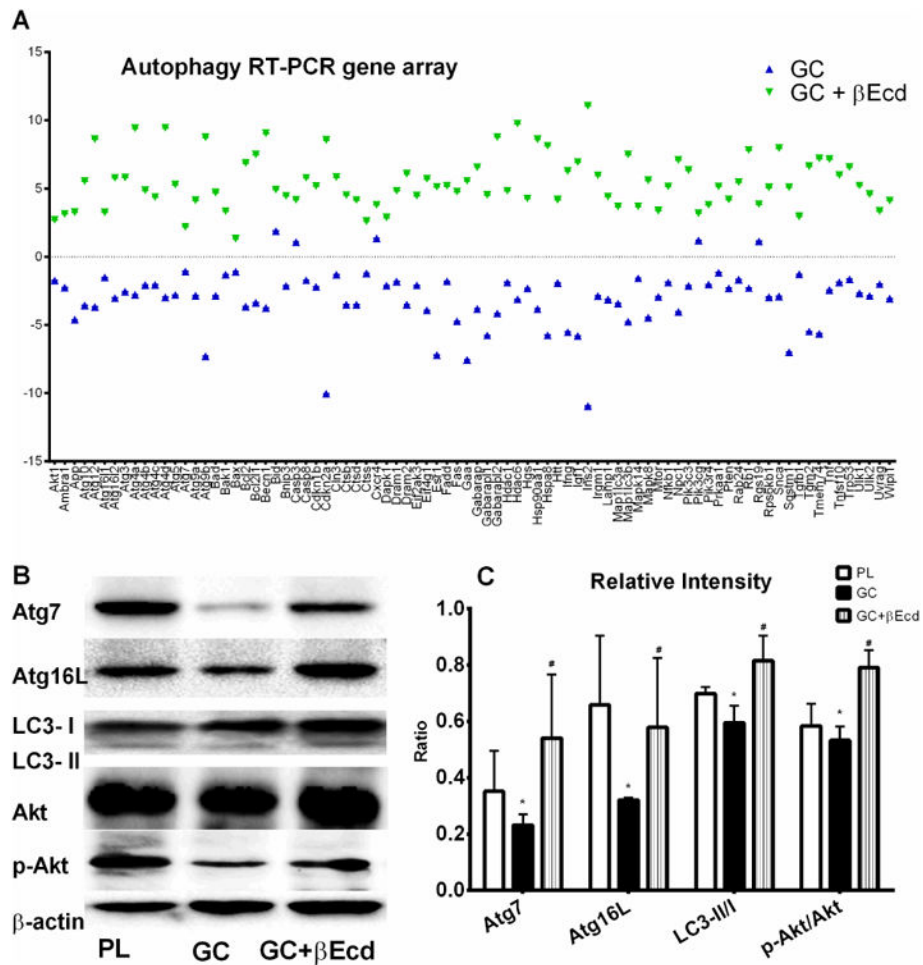


Figure 6.

GC decreased while β Ecd activated autophagy in bone. (A) RNA was extracted from the tibial shafts of PL, GC or GC+ β Ecd treated mice at day 21. The focus RT-PCR gene array for autophagy was performed. RT-PCR data was expressed as fold changes from the PL group. (B) Proteins were extracted from the distal tibiae in animals treated with PL, GC or GC + β Ecd. Western blots were performed for Atg7, Atg16L, LC3I/II, total Akt and p-Akt (S473). (C) Relative band intensity measurements on B (n=3/group). *: $p < 0.05$ vs. PL; #, $p < 0.05$ vs. GC.

Table 1

Serum hormone levels and bone turnover measurements

	Hormone panel				Bone panel			
	Cortisol (ng/ml)	Leptin (ug/ml)	Insulin (pg/ml)	OC (ug/ml)	OPG (ug/ml)	CTX-1 (ug/ml)		
PL	24.4 ±4.4	5.57 ±4.5	236 ±27	12.8± 1.7	1.05 ±0.5	19.7 ±4.1		
PL+βEcd	23.4 ± 3.6	4.02 ± 3.3	233 ± 84	16.1 ±2.7*	1.14 ±0.4	31.3 ±6.7*		
GC	17.5 ±3.2	3.47 ± 3.6	176 ±92	11.4 ±3.2	2.24 ±0.8	41.5 ±6.3*		
GC + βEcd	17.9 ±4.5	2.95 ±1.5	189±60	12.5 ±3.5	1.89 ±0.5	20.6 ±1.9#		

* *p* 0.05 vs. PL;# *p* 0.05 vs. GC.

Table 2

Effect of GC and β Ecd treatment on bone strength

	Vertebral compression test				Femoral bending test			
	Max Load (N)	Ultimate Stress (MPa)	Toughness (kJ/m ²)	Max Load (N)	Ultimate Stress (MPa)	Toughness (kJ/m ²)		
PL	20.2 ±3.7	3.32 ±0.7	0.51 ±0.1	13.1 ±2.1	182 ± 16	2.93 ± 0.9		
PL+ β Ecd	30.9 ±3.8*	4.03 ±0.9*	0.59 ±0.1	16.1 ±3.5*	259 ±49*	3.21 ±1.0		
GC	15.4 ±4.5*	2.70 ±0.7*	0.43 ±0.1*	13.4 ±2.5	175 ±23	2.24 ±0.4*		
GC + β Ecd	22.1 ±5.1*	3.54 ±1.2#	0.67 ±0.1#	15.6 ±1.8#	207 ± 6#	2.70 ±0.4#		

* $p < 0.05$ vs. PL;# $P < 0.05$ vs. GC.