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Prevention of glucocorticoid induced bone changes with beta-ecdysone ☆

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ABSTRACT

Beta-ecdysone (βEcd) 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 (GC) excess is associated with a decrease in bone formation, the purpose of this study was to determine if treatment with βEcd 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 (3.3 mg/kg/day) and treated with vehicle control or βEcd (0.5 mg/kg/day) 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 βEcd significantly increased bone formation on the endosteal and periosteal bone surfaces, and increased cortical bone mass were their controls to compare to GC alone. Concurrent treatment of βEcd and GC completely prevented the GC-induced reduction in BFR, trabecular bone volume and partially prevented cortical bone loss. *In vitro* studies determined that βEcd prevented the GC increase in autophagy of the bone marrow stromal cells as well as in whole bone. In summary, βEcd prevented GC induced changes in bone formation, bone cell viability and bone mass. Additional studies are warranted of βEcd for the treatment of GC induced bone loss.

Introduction

Glucocorticoids (GCs) are frequently used in clinical medicine to treat non-infectious inflammatory diseases. However, GCs 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 the 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 (βEcd) 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 pain and back pain. It has been shown that βEcd increases protein synthesis and reduces protein degradation in the skeletal muscle cells [24,25]. As it increases muscle weight in rodents [26–28], βEcd has been referred to as an "anabolic" naturally-derived supplement [29]. Additionally, βEcd is also found to stimulate mesenchymal stem cells' osteogenic differentiation but to inhibit their adipogenic differentiation [30]. βEcd is reported to increase the growth plate width in estrogen deficient rats and to have a marginal beneficial effect on the 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

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through multiple mechanisms [34], we seek to determine if βEcd can rescue the GC-suppression on bone formation. We have hypothesized that βEcd 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 βEcd treatment, and evaluate if autophagy is one of the mechanisms explaining the bone anabolic effect we observed for βEcd.

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 + βEcd (PL + βEcd 0.5 mg/kg, 5×/week); Group 3 was implanted with a prednisolone 5 mg/60 day slow-release pellet, which is equivalent to 3.3 mg/kg/day (GC), and Group 4 was implanted with prednisolone 5 mg/60 days slow-release pellet + βEcd (GC + βEcd 0.5 mg/kg, 5×/week). The mice were sacrificed after three weeks of treatments. The βEcd dose was based on publications on myogenesis and our *in vitro* experiments on osteogenesis and osteoclastogenesis using βEcd doses ranging from 10^{-3} to 10^{-9} M [25,26].

βEcd was purchased from Sigma-Aldrich (St. Louis, MO). Calcein (30 mg/kg) was injected to all mice for 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 μm. Bone samples were scanned at 70 kVp and 145 μA. 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 h, and then soaked in 30% sucrose in PBS at 4 °C for 8 h and then embedded in optimum cutting temperature compound. Eight micrometer 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 μm 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 surfaces 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 (BMSCs) were flashed out from long bones obtained from the 2-month-old male mice. For adipogenesis