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A novel flavonoid *C*-glucoside from *Ulmus wallichiana* preserves bone mineral density, microarchitecture and biomechanical properties in the presence of glucocorticoid by promoting osteoblast survival: A comparative study with human parathyroid hormone

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ABSTRACT

Purpose: 6-*C*-β-D-glucopyranosyl-(2S,3S)-(+)-5,7,3′,4′-tetrahydroxydihydroflavonol (GTDF) is a novel compound isolated from *Ulmus wallichiana*, reported to have bone anabolic action in ovariectomized rats. Here, we studied the effect of GTDF in glucocorticoid (GC)-induced bone loss and its mode of action.
Methods: Osteoblasts were cultured from rat calvaria or bone marrow to study apoptosis and differentiation by dexamethasone (Dex), methylprednisolone (MP), GTDF, quercetin and rutin. Female Sprague Dawley rats were treated with Dex or MP with or without GTDF or PTH. Efficacy was evaluated by bone microarchitecture using microcomputed tomography, determination of new bone formation by fluorescent labeling of bone and osteoblast apoptosis by co-labeling bone sections with Runx-2 and TUNEL. Serum osteocalcin was determined by ELISA.
Results: GTDF preserved trabecular and cortical bones in the presence of Dex and MP and mitigated the MP-mediated suppression of serum osteocalcin. Co-administration of GTDF to MP rats increased mineral apposition, bone formation rates, bone biomechanical strength, reduced osteoblast apoptosis and increased osteogenic differentiation of bone marrow stromal cells compared to MP group, suggesting *in vivo* osteogenic effect of GTDF. These effects of GTDF were to a great extent comparable to PTH. GTDF prevented GC-induced osteoblast apoptosis by inhibiting p53 expression and acetylation, and activation of AKT but did not influence transactivation of GC receptor (GR).
Conclusions: GTDF protects against GC-induced bone loss by promoting osteoblast survival through p53 inhibition and activation of AKT pathways but not as a GR antagonist. GTDF has the potential in the management of GC-induced osteopenia.

Abbreviations: GTDF, 6-*C*-β-D-glucopyranosyl-(2S,3S)-(+)-5,7,3′,4′- tetrahydroxydihydroflavonol; Dex, dexamethasone; MP, methyl prednisolone; Dox, doxorubicin; GR, glucocorticoids receptor; Runx-2, runt related transcription factor2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; BMD, bone and mineral density; TFSP, tibia-fibular separation point; μCT, microcomputed tomography; Tb.N, trabecular number; BV/TV, bone volume/trabecular volume; Tb.sp, trabecular separation; Tb.th, trabecular thickness; Tb.pf, trabecular pattern factor; SMI, structure model index; Conn.D, connection density; DA, degree of anisotropy; MAR, mineral appositional rate; BFR/BS, bone forming rate/bone surface; B.Ar, cortical mean cross-sectional area; Cs.Th, cortical thickness.

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Introduction

Glucocorticoids (GCs) are naturally produced steroid hormones or synthetic compounds [dexamethasone (Dex) and methylprednisolone (MP)] that are widely used for anti-inflammatory and immunosuppressive therapy (Schacke et al. 2002). Most dramatic side effects of long-term GC excess include bone loss and development of osteoporosis. GC therapy results in a rapid loss of bone mineral density (BMD), an important predictor of the risk of fracture (Mazziotti et al. 2006). The rate of loss is greatest in the first year of therapy and may be as high as 30% in the first six months (Bouvard et al. 2009). Bone loss occurs at both cortical and cancellous sites, and is associated with fractures of the hip, vertebrae, pelvis, forearm and ribs. The pathophysiological mechanism of GC-induced osteoporosis (GIO) includes suppression of bone formation and stimulation of osteoclastic bone resorption (Canalis et al. 2007; Weinstein et al. 2002). At the molecular levels, GCs modulate the transcription of various genes responsible for the synthesis of matrix constituents by osteoblasts, such as type 1 collagen and osteocalcin. GC shortens the lifespan of osteoblasts and osteocytes (O'Brien et al. 2004; Wang et al. 2002). In addition, GCs may promote osteoclast survival by lowering osteoprotegerin-to-receptor activator of nuclear kappa B ligand (RANKL) ratio in osteoblasts (Hofbauer et al. 1999).

Bisphosphonates are considered as the first-line of treatment for GIO (Amin et al. 2002). Bisphosphonate treatment is recommended for the duration of GC treatment as data demonstrate that fracture risk is highest during the time of GC exposure. The beneficial actions of bisphosphonates in GC-induced osteoporosis are attributed to their anti-resorptive effect (Carpinteri et al. 2010). An anabolic mode of treatment of GIO would rather have promise since the disorder is primarily one of reduced bone formation. Intermittent parathyroid hormone (PTH) administration is an attractive candidate therapy because it remains the only anabolic agent available for clinical use for new bone formation in postmenopausal osteoporosis. PTH exerts its anabolic effect by increasing proliferation and differentiation of osteoprogenitors (Kostenuik et al. 1999; Nishida et al. 1994), conversion of bone-lining cells to active osteoblasts (Dobnig and Turner 1995, 1997), and osteogenesis of mesenchymal stem cells at the expense of adipogenesis (Kulkarni et al. 2007; Rickard et al. 2006). Inhibition of osteoblast apoptosis also appears to contribute to the anabolic action of PTH, and the hormone is currently undergoing clinical trials to determine its efficacy in GIO.

Flavonols are a class of flavonoids with 3-hydroxy-2-phenylchromen-4-one backbone. In animal models, several flavonols (aglycone as well as glycoside forms) have been reported to counteract the bone deleterious effects of estrogen deficiency without uterotrophic effect (Bitto et al. 2009; Horcajada-Molteni et al. 2000). Recently, we showed that 2S,3S-2,3 dihydroquercetin-*C*-glucoside [IUPAC: 6-*C*-β-D-glucopyranosyl-(2S,3S)-(+)-5,7,3′,4′-tetrahydroxydihydroflavonol. (GTDF)], a novel analog of quercetin isolated from *Ulmus wallichiana* (Rawat et al. 2009), has bone anabolic action in osteopenic OVx rats (Sharan et al. 2011). Furthermore, positive skeletal effect of GTDF in OVx rats is devoid of uterotrophic response (Sharan et al. 2010). At the cellular level, one of the mechanisms by which GTDF exerted osteogenic effect was by promoting osteoblast survival (Sharan et al. 2011). Because, GC induces osteoblast apoptosis that leads to suppression of bone formation and thus forms the central feature of GIO pathogenesis (Canalis et al. 2007), we surmised that GTDF could address GIO pathophysiology. Accordingly, the effect of GTDF was evaluated in validated rodent models of GC excess by using BMD, dynamic- and static histomorphometries, and biomechanical strength measurements. The proof-of-concept that GTDF has anti-apoptotic effect on osteoblasts in GIO bones was evaluated. Intermittent injection of PTH was used as a reference therapy for evaluating bone anabolic action (Sato et al. 2004). Because, GCs modulate p53 (Li et al. 2009, 2012) and AKT (Almeida et al. 2011) pathways in osteoblast, we studied the effect of GTDF on these pathways as well as GC receptor (GR) activation.

Materials and methods

Materials

Cell culture media, supplements and fine chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). We procured Annexin V-FITC apoptosis detection kit and Polyvinylidenedifluoride (PVDF) membrane from Millipore (Temecula, CA); HTRF Sirt-1 deacetylation from Cisbio bioassays (Parc Marcel Boiteux, France); antibodies from Cell Signaling Technology (Beverly, MA); human PTH (1–34) from Calbiochem (La Jolla, CA, USA); TUNEL kit from Roche Applied science (Indianapolis, USA); Vectashield from Vector Laboratories (Burlingame, CA) and osteocalcin ELISA kit from Immunodiagnostic Systems Inc (Tyne & Wear, UK). U2OS cells were obtained from American Type Culture Collection (Manassas, VA).

Animals

Animal care and experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC). Female Sprague Dawley (SD) rats (180 ± 20 g) and new born pups (both sexes) were obtained from the National Laboratory Animal Centre, CSIR-CDRI. Animals were kept in a 12 h light–dark cycle, with controlled temperature (22–24 °C), humidity (50–60%) and free access to standard rodent food and water.

In vitro studies

Culture of calvarial osteoblasts

Rat calvarial osteoblasts (RCO) were obtained using the previously published protocol of sequential digestion (Gautam et al. 2011). Cells from second to fifth digestions were collected, centrifuged, resuspended and plated in T-25 cm^2 flask in α-MEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Cell viability assay

RCO at 70–80% confluence, were incubated in reduced serum (0.5% FBS) medium for 2 h, and then pre-treated with vehicle, GTDF, quercetin or rutin (100 nM) for 30 min followed by Dex or MP (1 μM) treatments. After 48 h, cell viability was measured to determine the viability of RCOs using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit.

Apoptosis assay

RCO were grown to 70–80% confluence, followed by serum withdrawal for 2 h and treatment with vehicle, Dex (100 nM), GTDF (100 nM), GTDF + Dex or CPFTH (10 μM) for 24 h. Annexin V-FITC apoptosis detection kit (Millipore, Temecula, CA) was used to measure apoptosis through FL1-H channel (Annexin V) FL2-H channel (PI) using a Becton Dickinson FacsCalibur flow cytometer (Franklin Lakes, NJ), according to manufacturer's instructions.

Sirtuin 1 activity assay

This was performed in cell free system using HTRF Sirt-1 deacetylaion kit following manufacturer's instructions.

Transient transfection assays

U2OS cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS. Transient transfection-based