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Detrimental effects of atherogenic and high fat diet on bone and aortic calcification rescued by an isoflavonoid Caviunin β-D-glucopyranoside

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ABSTRACT

Objective: Atherogenic diet (AD) and high fat diet (HFD) cause deleterious effect on bone micro-architecture and this phenomenon prompts aortic calcification. This study aims to show the effects of Caviunin β-D-glucopyranoside (CAFG), against bone loss and its associated aortic calcification in presence of AD and HFD challenged diets.
Methods: Five groups of C57BL/6 male mice with 8 animals in each group, comprising of chow, AD, HFD, AD + CAFG and HFD + CAFG were fed with respective diets for 16 weeks. At the end of the treatment period, preventive effects of CAFG on bone tissue were analyzed by assessing the osteogenic potential of bone marrow cells, bone micro-architecture, ability of new bone formation and histomorphometry studies. Aortic calcification was assessed by transcription and translation analysis of osteogenic key markers in aortic tissue and assessment of aortic endothelial function. Plasma lipid profiling was done to assess the effects of diets as its role in both bone loss and aortic calcification.
Results: Bone marrow stromal cell (BMSC's) dynamics showed that AD and HFD decreased osteoblast number that led to bone loss, deterioration in bone micro-architecture with up-regulated bone resorptive genes that lead to increase in aortic calcification. CAFG treatment rescued the bone health by modulating BMSC's towards osteogenic lineage. It increased the osteogenic gene expression with simultaneous decrease in osteoclastic genes thus stabilized the receptor activator of nuclear factor-kappa-B ligand/osteoprotegerin ratio that eventually reduced the amount of calcification in aorta. Biochemical studies showed that CAFG reduced the TC, TG and LDL-C content with no marked changes in HDL-C. Moreover, CAFG decreased the osteogenic key markers in the aortic tissue and enhanced endothelial function.
Conclusion: Overall, this study indicates that CAFG protected against physiologically challenged diet induced bone loss with associated vascular calcification in mice. Moreover, data revealed that atherogenic diet is more detrimental as compared to the excess fatty acid diet to the bone and aorta.

1. Introduction

Osteoporosis is an age related disease characterized by low bone mass and increased fragility that leads to increased fracture risks [1]. It is commonly observed that the decrease in bone mineralization increases vulnerability to atherosclerosis and this process of calcification in vascular tissue is a highly regulated process of mineralization, which involves cellular and molecular

Abbreviations: AD, atherogenic diet; HFD, high fat diet; CAFG, Caviunin 7-O-[β-D-apiofuranosyl-(1-6)-β-D-glucopyranoside; BMSC's, bone marrow stromal cell; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; RANKL, receptor activator of nuclear factor-kappa-B ligand; OPG, osteoprotegerin; BV/TV, bone volume/tissue volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Pf, trabecular bone pattern factors; SMI, structure model index; MAR, mineral appositional rate; MS/BS, mineralizing surface; pBFR/BS, peripheral bone formation rate; TRAP, tartrate-resistant acid phosphatase; TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high-density lipoproteins- cholesterol; TG, total triglycerides; ALP, alkaline phosphatase; RUNX2, Runt-related transcription factor-2; BMP2, bone morphogenetic protein-2; COL1, collagen type-1; OPN, osteopontin; MSX2, Msh Homeobox-2.

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signalling pathways as that of normal osteogenesis [2,3]. Clinical data show that patients with osteoporosis also often suffer from vascular calcification, this has further been confirmed by knockout mice studies which show common pathogenesis of osteoporosis and atherosclerosis [4]. In our earlier studies, we have observed that obesity induced by high fat diet (HFD) treatment has deleterious effects on bone micro-architecture in growing male mice by directing progenitor bone marrow stromal cell (BMSC's) to undergo adipogenic instead of osteogenic differentiation [5]. Many recent as well as past studies confirm the biological basis for linking the sides of osteoporosis and atherosclerosis using therapies that may enhance bone density and diminish atherogenesis at the same time. Statins are one such class of drugs employed against both atherosclerosis and bone related disorders [6,7]. Bisphosphonates [8,9] and Parathyroid hormone (PTH) treatment have also been found to increase the bone mineral density and suppress aortic valve calcification simultaneously [10]. Earlier studies suggest that impaired bone metabolism and progression of vascular calcification have common pathological markers like, the markers of inflammation Interleukin 6 (IL-6) and Tumor necrosis factor-α (TNF-α), receptor activator of nuclear factor-kappa-B ligand (RANKL)/RANK/osteoprotegerin (OPG) and bone marrow adipogenesis which are considered as risk factors directly for both bone and aortic tissue [11–13]. Currently, phytochemicals are receiving increased attention due to their health-associated benefits along with beneficial effects on bone health [13–17]. In our previous study, we have established that Caviunin 7-O-[β-D-apiofuranosyl-(1-6)-β-D-glucopyranoside] [(CAFG), a novel isoflavonoid isolated from leaves of *Dalbergia sissoo* improved trabecular microarchitecture of the long bones, inhibited osteoclast activation and decreased bone turnover markers when supplemented orally to rodents [18]. These beneficial effects of CAFG on bone health prompted us to study its effect in cholesterol excess and high fatty acid diet and assess if CAFG could also simultaneously impact the bone loss and vascular calcification positively.

2. Material and methods

2.1. Chemicals

Cell culture media Dulbecco's Modified Eagle Medium (DMEM), minimum essential media (MEM) and supplements fetal bovine serum (FBS), Insulin, Dexamethasone (DEX), indomethacin, 3-isobutyl-1-methylxanthine (IBMX), β-glycerophosphate (β-GP) were purchased from Invitrogen (Carlsbad, CA, USA) and Sigma (St. Louis, MO, USA). All fine chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). AD [Research diet, D12109] and HFD [Research diet, D12492] were purchased from the Research diet (New Brunswick, USA). TRIzol reagent and cDNA synthesis kit were purchased from Life Technologies (Thermo Scientific, Massachusetts, USA). SYBR green kit was purchased from genetix (Puregene, Genetix New Delhi, India).

2.2. Ethics, animal model and diets/treatment

Animal experiments were conducted in accordance with regulations of the Institutional Animal Ethical Committee (Approval no.IAEC/2013/16) at the National Laboratory of Animal Sciences (NLAC) of Central Drug Research Institute Lucknow India. Forty male C57BL/6 mice, of weight approx. 20 g and 8 weeks old were obtained for the study (n = 8 per group). All mice were individually housed at 21 °C in 12-h light: 12-h dark cycles. Mice were randomly divided into 5 groups and were kept on standard chow diet (CD), AD, HFD, AD + CAFG and HFD + CAFG for 16 weeks [19]. Detailed compositions of different diets are shown in Supp. Table 1B. All groups received the equal amount of food diet (8.0 g/animal for all types of diets i.e.CD, AD and HFD) in the morning and in the next day; the leftover food amount was subtracted from food amount given in the previous day to find the exact food consumption/day/animal. CAFG was administrated orally for 16 weeks through gavages. CAFG (1.0 mg kg^{-1} day^{-1} body weight) was chosen for dosing on the basis of our previous studies [18,20]. Structure of CAFG is showing in Fig. 1A. Body weights were measured weekly.

2.3. Differentiation of osteoblasts and adipocytes from BMSC's

At the end of 16 weeks of treatment, long bones of all mice groups were excised aseptically. BMSC's were harvested and cultured as previously described [21]. Briefly, 3 animals from each group were sacrificed and femur/tibia bones were removed under sterile conditions. After removal of the bone heads, the marrow was collected by flushing repeatedly through the shafts with a syringe containing DMEM supplemented with antibiotics and 10% heat-inactivated FBS. Aggregates were removed from the cell suspension by sieving through a 70 μm cell strainer. Cell numbers were determined using trypan blue staining. For osteoblasts differentiations, 2×10^6 BMSC's were plated in α-MEM supplemented with 10% FBS, 10^{-7} M DEX, 50 μg/ml ascorbic acid and 10 mM β-GP. Released BMSC's were cultured for 21 days. After 21 days, cells were fixed in 4% paraformaldehyde, stained with 40 mM Alizarin Red-S and read at 405 nm in plate reader (spectra max paradigm, molecular devices, USA) to measure nascent calcium. For adipocytes differentiation, 2×10^6 BMSC's were seeded in 12-well plates in adipogenic differentiation medium comprising of DMEM containing 10% FBS, 1.0 μM DEX, 0.5 mM IBMX, 100 μM indomethacin and insulin (10 μg/ml). After 18 days of culture, the cells were fixed in 4.0% paraformaldehyde and stained with Oil Red-O. For extraction of Oil Red-O stain, 0.5 ml of 70% isopropanol was added to the stained cells at 37 °C for 20 min [22]. The extracted stain was quantified by the plate reader at 490 nm [5].

2.4. Micro-computed tomography analysis of trabecular bone

Micro-CT analyses of excised bones were carried out using the SkyScan 1076 μ-CT scanner (Aartselaar, Belgium). The bones were first fixed in 4.0% paraformaldehyde and then stored in 70% ethanol for scanning. Bones were scanned at voxel size of 9.0 μm, at a voltage of 50 kV, a current of 152 mA, by using a 0.5 mm aluminium plate (for removing signal noise), over 360°, 0.6° rotation step with full width. After scanning, reconstructions of bones were carried out by Sky Scan Nrecon software. The trabecular bone part of distal femur, proximal tibial metaphysis and L5 vertebrae was extracted by drawing ellipsoid contours with the CT analyzer software. 3-D images of trabecular region of bones were drawn by CT volume software by selecting ROI and 2-D drawn by Data-viewer software. The trabecular bone volume/tissue volume BV/TV (%), trabecular number (Tb.N) (mm^{-1}), trabecular separation (Tb.Sp) (mm), trabecular bone pattern factors (Tb.pf) (mm^{-1}) and structure model index (SMI) were directly measured by BATMAN software provided with the μ-CT instrument [23,24].

2.5. Bone strength study

During autopsy, femurs were dissected and separated from adjacent tissue, cleaned and used for physical measurements and mechanical testing. All specimens were tested under load applied at a constant rate of 20 mm/min. This test predominantly measured cortical bone strength parameters like power, energy