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Kaempferol has osteogenic effect in ovariectomized adult Sprague–Dawley rats

Ritu Trivedi[a,*,1], Sudhir Kumar[a,1], Avinash Kumar[a], Jawed A. Siddiqui[a], Gaurav Swarnkar[a], Varsha Gupta[b], Amruta Kendurker[c], Anil Kumar Dwivedi[b], Jose R. Romero[d], Naibedya Chattopadhyay[a]

[a] Division of Endocrinology, Central Drug Research Institute, Chattar Manzil, Lucknow, India
[b] Division of Pharmaceutics, Central Drug Research Institute, Chattar Manzil, Lucknow, India
[c] Division of Pharmacokinetics, Central Drug Research Institute, Chattar Manzil, Lucknow, India
[d] Division of Endocrinology, Metabolism & Hypertension, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Kaempferol (K), a flavonol, is known to have anti-osteoclastogenic effect. We here show that K, from 0.2 to 5.0 μM, increased mineralized nodules in rat primary osteoblasts. K also significantly attenuated adipocyte formation from bone marrow cells (BMCs). A single oral dose of 1 mg/kg body weight of K in *Sprague–Dawley* (180–200 g) rats resulted in a peak serum level of 2.04 ± 0.8 nM in 30 min (T_{max}), suggesting its rapid absorption. The C_{max} of K in bone marrow was 0.684 nM after 90 min. Rats were ovariectomized (OVx) along with sham-operated rats and left for 4 weeks. Daily oral administration of K (5 mg/kg body weight) was then started to one group of OVx rats, and continued for 10 weeks. K levels were found to be 0.311 and 0.838 nM at the end of 4 and 10 weeks, respectively. K exhibited no estrogenicity at the uterine level. The K-treated group exhibited significantly higher bone mineral density (BMD) in the trabecular regions (femur neck, proximal tibia and vertebrae) and lower serum ALP (bone turnover marker) compared with the OVx rats. The compressive energy of the vertebrae was significantly higher in the OVx + K-treated group compared with the OVx group. K treatment of OVx rats resulted in the increase in osteoprogenitor cells as well as inhibition of adipocyte differentiation from BMCs compared with the OVx group. Together we show that K is non-estrogenic *in vivo* and exerts bone anabolic activity with attendant inhibition of bone marrow adipogenesis.

1. Introduction

The use of flavonoids has been widely implicated in the alleviation of postmenopausal osteoporosis. Isoflavonoids such as genistein and daidzein, and flavonols such as quercetin (3,3′,4′,5,7-pentahydroxyflavone) in the form of rutin have been shown to prevent bone loss in ovariectomized (OVx) rats (Horcajada-Molteni et al., 2000). Kaempferol (K) [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one] is a flavonol that exhibits anti-osteoclastogenic activity *in vitro* (Wattel et al., 2003). K is highly abundant in *Ginkgo biloba* extracts, which is used as a popular nutraceutic (2003).

K acts directly on the precursors of osteoclastogenic cells as well as on the mature osteoclasts to inhibit their differentiation and induce apoptosis, respectively (Wattel et al., 2003). We have shown that K inhibits TNFα-induced secretion of key osteoclastogenic cytokines, MCP-1 and IL-6, and inhibits NF-κB signaling in osteoblasts (Pang et al., 2006). In addition, K has been shown to promote differentiation of osteoblast-like MG-63 cells and also to promote differentiation and mineralization of a mouse osteoblast cell line, MC3T3-E1 (Kim et al., 2003; Prouillet et al., 2004). However, no data are available on the effects of K on primary osteoblasts (calvarial or bone marrow-derived) and its *in vivo* efficacy in bone loss models such as estrogen-deficient rats (ovariectomized).

One of the prerequisites for anti-osteoporosis therapy is lack of estrogenicity of a given compound/agent. K has been shown to mediate anti-osteoclastogenic action (osteoclast apoptosis) and increase ALP production in MG-63 cells by estrogen receptor (ER); an estrogen antagonist, ICI 182782, blocked both the functions of K (Prouillet et al., 2004). Since 17β-estradiol was found to have no effect on TNFα-induced synthesis of IL-6 from osteoblast (Pang et al., 2006) while K inhibited it, which suggests that K may not have estrogen-like action in MC3T3-E1 cells. In addition, K was shown to have either weak or no estrogenic effect on the uterus of immature rats (Stroheker et al., 2002). Therefore, given its actions on osteoclasts and osteoblasts *in vitro* coupled with a lack of estrogenicity in

* Corresponding author.
E-mail address: ritu_pgi@yahoo.com (R. Trivedi).
[1] Both the authors contributed equally to this study.

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immature rats makes K an attractive compound to study its putative anti-osteoporotic activity *in vivo*.

Bioavailability of flavonoids is a subject of much debate, with the absorbability of glycoside versus aglycone forms being a central theme (Liu and Hu, 2007; Lotito and Frei, 2006). Pharmacokinetic data on flavonoids are deficient and for K they are completely lacking. The required micromolar levels of K raise concerns about achieving these levels in the serum.

We therefore sought to study the effects of K in primary osteoblasts (both calvarial and bone marrow cells (BMCs)) and on bone marrow adipogenesis, as an increase in the latter has been shown to promote bone loss. We also studied the effect of K *in vivo* in a rat model of bone loss (OVx rats) and determined its bioavailability. Finally, using *ex vivo* bone marrow cultures, we determined the effects of K on osteoprogenitor cells and adipogenesis. Our data suggest that K has *in vivo* efficacy in a rat model of bone loss and raise the possibility of its therapeutic use.

2. Materials and methods

2.1. Reagents and chemicals

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). All fine chemicals including kaempferol were purchased from Sigma–Aldrich (St. Louis, MO). HPLC grade acetonitrile was obtained from Merck India Ltd. (Mumbai, India).

2.2. Culture of calvarial osteoblasts

Rat calvarial osteoblasts were obtained following our previously published protocol of sequential digestion (Wong and Cohn, 1975). Briefly, calvaria from ten to twelve 1- to 2-day-old *Sprague–Dawley* rats (both sexes) were pooled. After surgical isolation from the skull and the removal of sutures and adherent mesenchymal tissues, calvaria were subjected to five sequential (10–15 min) digestions at 37 °C in a solution containing 0.1% dispase and 0.1% collagenase P. Cells released from the second to fifth digestions were collected, centrifuged, resuspended, and plated in T-25 cm^2 flasks in α-MEM containing 10% FCS and 1% penicillin/streptomycin (complete growth medium).

2.3. Mineralization of calvarial osteoblasts

Calvarial osteoblasts cultured until 80% confluence were trypsinized and plated in the differentiation medium (2.5×10^4 cells/well in 12-well plate), consisting of complete growth medium with ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM). The medium was changed every alternate day up to 21 days. A 10 mM stock solution of K was made in DMSO. Treatment with K at various concentrations was given in such a manner that the final concentration of DMSO would not exceed 0.01%. *In vitro* treatments were made with 0.01% DMSO which served vehicle control. The treatment group contained osteoblast differentiation medium with K (0.2, 1 and 5 μM). At the end of the experiment, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were stained with 40 mM (pH 4.5) Alizarin Red-S for 30 min followed by washing with water (Prabhakar et al., 1998).

For quantification of staining, 800 μl of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped from the plate with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5-ml tube. After vortexing for 30 s, the slurry was overlaid with 500 μl mineral oil (Sigma–Aldrich), heated to exactly 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000 × *g* for 15 min and 500 μl of the supernatant was removed to a new tube. Then 200 μl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. OD (405 nm) of 150 μl aliquots of the supernatant were measured in 96-well format using opaque-walled, transparent-bottomed plates (Gregory et al., 2004).

2.4. Mineralization of BMCs

BMCs from female *Sprague–Dawley* rats weighing ~40 g were isolated and cultures prepared according to a previously published protocol (Maniatopoulos et al., 1988). Briefly, the femora were excised aseptically, cleaned of soft tissues, and washed 3 times, 15 min each, in a culture medium containing 10 times the usual concentration of antibiotics as mentioned above. The epiphyses of femora were cut off and the marrow flushed out in 20 ml of culture medium consisting of α-MEM, supplemented with 15% fetal bovine serum, 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Released BMCs were collected and plated (2×10^6 cells/well of 12-well plate for mineralization assay and 10^6 cells/well of 48-well plate for ALP assay) in the culture medium, consisting of α-MEM, supplemented with 15% fetal bovine serum, 10^{-7} M dexamethazone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Cells were cultured with and without K for 11 (ALP) and 21 (mineralization) days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 48 h. After 21 days, the attached cells were fixed in 4% formaldehyde for 20 min at room temperature and rinsed once in PBS. After fixation, the specimens were processed for staining with 40 mM Alizarin Red-S, which stains areas rich in nascent calcium. Determination of ALP activity by osteoblast cells was done after 11 days of culture.

2.5. Induction of adipogenic differentiation from BMCs

For adipogenic differentiation, 2×10^6 BMCs were seeded in 24-well plates and cultured in an adipogenic medium consisting of DMEM containing 10% FCS (complete growth medium) plus 10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 50 mM indomethacin up to 7 days. On day 3, complete growth medium containing insulin 10 μg/ml was added. This treatment continued for 21 days and the medium was changed every 3 days. The treatment group contained a similar medium as the K group (0.2, 1, and 5 μM). After 21 days of culture, the cells were fixed in 4% paraformaldehyde and stained with Oil Red O. To quantify the incorporation of lipid, the area stained with Oil Red O was measured by taking photomicrographs (Heim et al., 2002).

For extraction of Oil Red O stain, 500 μl of 70% propanol was added to the stained cells and left at 37 °C for 20 min (Heim et al., 2002). The extracted stain was quantified by the ELISA plate reader at 490 nm.

2.6. In vivo experiments

The study was conducted in accordance with current legislation on animal experiments [Institutional Animal Ethical Committee (IAEC)] at C.D.R.I. Thirty female *Sprague–Dawley* rats weighing 180–200 g were taken for the study ($n = 10$/group). Twenty rats were bilaterally ovariectomized. The other 10 rats were exposed to a sham surgical procedure. All rats were individually housed at 21 °C, in 12-h light:12-h dark cycles. Normal chow diet and water were provided *ad libitum*. The rats were left for 4 weeks to develop osteopenia.

After 4 weeks, K treatment in the form of gavage (5.0 mg/kg body weight or 17.5 μM by oral route in 20% ethanol) was given daily for 10 weeks. Equal numbers of OVx and sham operated rats served as the control, and were given vehicle (20% ethanol). The rats were weighed each week.

At the end of 10 weeks of gavage, the rats were caged individually in plastic cages fitted with steel mesh for a total period of 48 h preceding autopsy and had free access to normal chow diet and water for the first 24 h of acclimatization. During the next 24 h, animals received only water *ad libitum*. Twenty-four hour fasting urine samples were collected in fresh containers centrifuged at 2000 rpm at room temperature and stored at −20 °C until analyzed. After urine collection the rats were euthanized. At autopsy, blood samples were collected by cardiac puncture in tubes, and serum samples collected and frozen until analysis. Uteri were carefully excised, gently blotted, weighed, and fixed for histology and histomorphometry. About 5-mm pieces from the middle segment of each uterus were dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin wax using standard procedures. Representative transverse sections (5 μm) were stained with haematoxylin and eosin. Photomicrographs of sections were obtained using a Leica DC 300 camera and Leica IM50 Image Acquisition software fitted to a Leica DMLB microscope. Histomorphometric measurements were done using Leica Qwin-Semiautomatic image Analysis software.

The bone marrow was harvested for *ex vivo* experiments. Left and right femurs were dissected and separated from adjacent tissue, cleaned, and used for physical measurements mechanical testing and bone mineral density (BMD) measurement.

2.7. Determination of K in serum and bone marrow

K in 20% ethanol was administered by gavage method (1 mg/kg). Following administration, the rats were sacrificed at 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, and 24 h to obtain serum and bone marrow. Three animals were used for each time point. The blank blood and bone marrow sample was obtained from the animals given no K treatment.

1 ml of acetonitrile was added to 0.5 ml of plasma or bone marrow samples. The resulting solution was thoroughly vortex-mixed for 2 min. After centrifugation at 3000 × *g* for 10 min, the supernatant layer was transferred into a clean test tube, concentrated to dryness under vacuum, reconstituted in 50 μl of acetonitrile. Bone marrow cells were reconstituted in 50 μl of methanol and 20 μl of serum or bone marrow cells were injected into the HPLC system for analysis.

Concentration of K was measured by HPLC. A stock solution (206 μg/ml) of K was prepared in methanol. A series of standard solutions with concentrations in the range of 0.515–12.36 μg/ml were obtained by serial dilution. 50 μl of standard solutions was added to 250 μl of blank plasma to prepare standard calibration samples. The final standard plasma and bone marrow concentrations were 51.5–1236 ng/ml