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## Differential activity of kaempferol and quercetin in attenuating tumor necrosis factor receptor family signaling in bone cells

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### ABSTRACT

Increasing data from epidemiological and in vitro studies show that the isoflavonoids, genistein and daidzein, and the flavonols, quercetin and kaempferol, are protective against postmenopausal bone loss. However, the physiological mechanisms for these effects are not well understood. We now report that kaempferol exerts profound antiosteoclastogenic effects by acting on both osteoblasts and osteoclasts. Kaempferol but not quercetin dose-dependently inhibited tumor necrosis factor  $\alpha$  (TNF $\alpha$ )-induced production of the osteoclastogenic cytokines interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1/CCL2) in osteoblasts. The effect on IL-6 was posttranscriptional, whereas kaempferol reduced MCP-1 mRNA levels. In addition, in mouse primary calvarial osteoblasts, kaempferol but not quercetin blocked TNF $\alpha$ -induced translocation of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) subunit p65 from the cytoplasm to the nucleus. However, TNF $\alpha$ -stimulated intracellular ROS production was unaltered by kaempferol. In RAW264.7 cells, a monocyte/macrophage precursor for osteoclasts, both kaempferol and quercetin dose-dependently inhibited the receptor activator of NF- $\kappa$ B ligand (RANKL)-induced immediate-early oncogene c-fos expression at 6 h. After 3–5 days, both flavonols robustly inhibited RANKL-induced expression of the osteoclastic differentiation markers, RANK and calcitonin receptor. Consistent with down regulation of these osteoclastic differentiation markers, both flavonols strongly attenuated the RANKL-induced formation of multinucleated osteoclasts. However, kaempferol was more potent than quercetin in inhibiting RANKL-stimulated effects on RAW264.7 cells. Thus, our data indicate that kaempferol exerts profound antiosteoclastogenic effects by specifically antagonizing TNF receptor family action on bone cells at two distinct levels, by disrupting production of osteoclastogenic cytokines from osteoblasts and attenuating osteoclast precursor cell differentiation.

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## 1. Introduction

Bone remodeling is a physiological phenomenon that involves bone resorption by osteoclasts and bone formation by osteoblast with no net bone loss. Osteoporosis is a metabolic bone disorder where bone loss takes place due to an excess of osteoclastic bone resorption over osteoblastic bone formation. Osteoclasts are derived from hematopoietic progenitors of the monocyte-macrophage lineage. The tumor necrosis factor (TNF) family of receptors and ligands are the most prominent and important factors in osteoclastogenesis (for review [1,2]). Osteoclast formation, activation, and survival require the interaction of receptor activators of nuclear kappaB (RANK) and RANK ligand (RANKL), a conserved member of the TNF receptor and TNF ligand families, respectively [2]. Osteoblasts, which are mesenchymal cell-derived, play a direct and essential role in the overall osteoclast function within the bone microenvironment, which involves the regulation of osteoprotegerin/RANKL/RANK triad [3,4].

TNF $\alpha$ , produced mainly by activated macrophages, bone marrow stromal cells, and monocytes, is a potent osteoclastogenic cytokine promoting bone resorption [5,6]. TNF $\alpha$  stimulates osteoclast differentiation [7,8] and formation of multinucleated cells [9] *in vitro* and *in vivo* [10,11]. TNF $\alpha$  also enhances osteoclastic activity by stimulating osteoblasts to produce a variety of osteoclastogenic cytokines such as interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) [12,13]. Thus, these TNF family members, together with RANKL, represent the most potent osteoclastogenic cytokines (for review [1,2]) that regulate physiological bone remodeling and have been implicated in the pathogenesis of numerous bone disorders including osteoporosis.

Flavonoids have multiple beneficial biological activities owing to their anti-oxidant, anti-inflammatory and estrogenic effects [14,15]. Estrogen exerts bone anabolic effects by promoting key osteoblast functions and inhibiting osteoclastogenesis (for review [16]). Estrogen actions are predominantly mediated by its high affinity nuclear receptors and many of the flavonoids are considered phytoestrogens owing to their ability to bind and signal via estrogen receptors (for review [17]). In the context of bone health, recent reports indicate that the isoflavonoids genistein and daidzein significantly prevent bone loss in ovariectomized rats [18]. Similar effects were observed with the flavonol, quercetin (3,3',4',5,7-pentahydroxyflavone) (in the form of rutin) [19]. However, at the physiological level, very little is known about the mechanisms that mediate the prevention of bone loss by flavonoids. It is hypothesized that flavonoids such as quercetin would inhibit osteoclast function. Indeed, the commonly observed flavonols quercetin and kaempferol (3,4',5,7-tetrahydroxyflavone) have been shown to inhibit osteoclastic bone resorption and osteoclast apoptosis, probably via their estrogenic effect, as reported in a rabbit long bone osteoclast model [20].

We characterized the physiological mechanism by which kaempferol and quercetin inhibit bone resorption by examining the effects of TNF $\alpha$  and RANKL on osteoblast and osteoclast function. We report that kaempferol but not quercetin inhibits TNF $\alpha$ -induced production of the osteoclastogenic cytokines IL-6 and MCP-1 from the osteoblasts and blocks TNF $\alpha$ -induced NF- $\kappa$ B (p65) nuclear translocation in

these cells. In addition, we show that in RAW264.7 monocyte/macrophage cells both kaempferol and quercetin inhibit RANKL-induced expression of c-fos, which is required for osteoclast differentiation. Thus our report provides novel physiological mechanisms underlying the *in vivo* observation of prevention of ovariectomy-induced bone loss by the antiosteoclastogenic action of quercetin [19]. Furthermore, our results raise the possibility that kaempferol is a more potent antiosteoclastic agent *in vivo* due to its action on both osteoblasts and osteoclasts.

## 2. Materials and methods

### 2.1. Materials

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). Kaempferol and quercetin were purchased from Indofine Chemical Company Inc. (Belle Mead, NJ). Recombinant mouse TNF $\alpha$ , RANKL, and an ELISA kit for murine MCP-1 were purchased from R&D Systems (Minneapolis, MN). Mouse cytokine antibody array II and an IL-6 ELISA kit were purchased from Raybiotech Inc. (Norcross, GA).

### 2.2. Cell culture

The mouse calvarial osteoblast cell line MC3T3-E1 was purchased from American Type Culture Collection (Manassas, VA) and cultured in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Primary mouse calvarial osteoblasts were cultured from 1-day-old mice (Balb/c) as previously described [21]. Humane handling of mice was carried out according to the guidelines of The Center for Animal Resources and Comparative Medicine at Harvard Medical School. Fifteen to 20 calvariae were harvested at room temperature. Briefly, cells were released by repeated digestion of the calvariae with 0.05% trypsin and 0.1% collagenase P. The cells from the first two digestions were discarded. The cells from the next three digestions were pooled and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated FBS and 1% penicillin-streptomycin in 5% CO<sub>2</sub> at 37 °C. After 3 days of culture, cells were serum-starved in DMEM (1% penicillin-streptomycin, and 0.2% BSA) for 4 h before experimentation. All experiments were done within 7 days of isolation of calvarial osteoblast, at which stage the cells are still proliferating. Routinely, all experiments on MC3T3-E1 cell line or primary osteoblasts were performed at no more than 80% confluency.

The RAW264.7 monocyte/macrophage mouse cell line was obtained from American Type Culture Collection and maintained in culture according to their instructions. Cells were cultured routinely in complete growth medium ( $\alpha$ -MEM containing 10% heat-inactivated FBS). To differentiate the cells to osteoclast-like cells, RAW264.7 cells were gently scraped and seeded in either 100-mm<sup>2</sup> plates or 24-well plates at a density of 250,000/plate or  $3 \times 10^3$  cells/well, respectively, and cultured for 5–6 days in complete growth medium plus 50 ng/ml recombinant murine RANKL. Medium was replaced on the third day, and cells were cultured for two more days, at

which point large numbers of multinucleated cells were observed. At the end of the culture period, tartrate-resistant acid phosphatase (TRAP)-positive cells were counted after staining with a leukocyte acid phosphatase kit (Sigma).

### 2.3. Mouse cytokine antibody array

MC3T3-E1 cells were cultured to 80% confluency in 6-well plates. Cells were serum-starved in  $\alpha$ -MEM containing 0.5% heat-inactivated FBS and 1% penicillin–streptomycin for 4 h. After serum-starvation, cells were incubated in the medium containing 2 ng/ml TNF $\alpha$  or TNF $\alpha$  + 20  $\mu$ M kaempferol for 18 h. The cytokine composition of the conditioned medium was determined by cytokine antibody array following the manufacturer's instructions. The array contained antibodies against CCL21/6Ckine, cutaneous T-cell attracting chemokine (CTACK/CCL27), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, -3, -4, -5, -6, -9, -10, -13, -17, IL-12p40p70, IL-12p70, interferon gamma (IFN- $\gamma$ ), keratinocyte-derived chemokine (KC/mouse homolog of human CXCL1), leptin, MCP-1/CCL2, MCP-5/CCL12, macrophage inflammatory protein-1 alpha (MIP-1 $\alpha$ /CCL3), MIP-2, MIP-3 $\beta$ /CCL19, regulated upon activation, normal T-cell expressed and secreted (RANTES/CCL5), stem cell factor/kit ligand (SCF), soluble TNF receptor I (sTNFRI), thymus and activation-regulated chemokine (TARC/CCL17), tissue inhibitor of metalloproteinase-1 (TIMP-1), TNF $\alpha$ , thrombopoietin, and vascular endothelial growth factor (VEGF).

### 2.4. NF- $\kappa$ B localization by immunofluorescence

Mouse primary calvarial osteoblasts were cultured on glass coverslips as described above. Before experimentation, cells were incubated with 0.5% serum containing  $\alpha$ -MEM for 4 h at 37 °C. For kaempferol and quercetin treatments, cells were incubated with 10  $\mu$ M of each compound for 10 min before TNF $\alpha$  treatment. Cells were then treated with or without 2 ng/ml TNF $\alpha$  and TNF $\alpha$  plus either 10  $\mu$ M kaempferol or quercetin for 1 h at 37 °C and fixed with 2% paraformaldehyde for 10 min at room temperature. Cells were washed twice with PBS and incubated for 10 min in 0.5% Triton X 100-PBS solution. Before

addition of the anti-p65 primary mouse antibody (Santa Cruz Biotech), calvarial osteoblasts were blocked for 30 min with 1% BSA + PBS solution at room temperature. Cells were then incubated overnight at 4 °C with the primary antibody (1:100 diluted in PBS containing 0.5% BSA). Coverslips were washed three times for 10 min and incubated for 1 h at room temperature with fluorescent AlexaFluor-488 goat anti-mouse IgG (H + L) (Molecular Probes) secondary antibody used at a 1:100 dilution in PBS. Coverslips were washed (three times 10 min) and mounted on slides using VECTASHIELD<sup>®</sup> before examination on a fluorescent microscope; 3–4 randomly chosen fields/coverslips were rated positive for nuclear translocation of NF- $\kappa$ B when NF- $\kappa$ B-labeled fluorescence of the nuclei exceeded that of the cytoplasm. Five coverslips for each treatment were thus counted, and data were expressed as percentage nuclear translocation over control.

### 2.5. Measurement of IL-6 and MCP-1 in cell culture supernatants

Primary cultures of osteoblasts or MC3T3-E1 cells were seeded in 24-well plates and cultured till they reached 70–80% confluence. The cells were starved in serum-free medium (DMEM containing 1% penicillin–streptomycin, and 0.2% BSA) for 4 h. After 18 h stimulation with various reagents supernatants were collected and analyzed for IL-6 and MCP-1 content with a colorimetric sandwich ELISA kit.

### 2.6. Quantitative real-time polymerase chain reaction (QPCR)

cDNA was synthesized from 2  $\mu$ g total RNA with the Omniscript reverse transcription kit (QIAGEN, Valencia, CA). SYBR green chemistry was used for quantitative determination of the mRNAs for IL-6, MCP-1, RANK, CTR, and c-fos with a housekeeping gene, cyclophilin A, following an optimized protocol [21]. The design of sense and antisense oligonucleotide primers was based on published cDNA sequences using the Primer Express (version 2.0.0, Applied Biosystems). Table 1 shows the primer sequences used. For real-time PCR, the cDNA was amplified with an ABI PRISM 7000 (PE Applied Biosystems). The double-stranded DNA-specific dye SYBR

**Table 1 – QPCR primers**

Gene name	Accession number	Primers
IL-6	NM_031168	TAG TCA ATT CCA GAA ACC GCT (F) GAA GTA GGG AAG GCC GTG GT (R)
MCP-1/CCL2	NM_011333	TGC ATC TGC CCT AAG GTC TTC (F) AAG TGC TTG AGG TGG TTG TGG (R)
RANK	NM_009399	GCC CAG TCT CAT CGT TCT GC (F) TAG CTG TCA GCG CTT TCC CT (R)
CTR	NM_007588	TCA GGA ACC ACG GAA TCC TC (F) ACA TTC AAG CGG ATG CGT CT (R)
c-Fos	NM_010234	GAC TAC GAG GCG TCA T (F) GAT CTG TCC GTC TCT AGT (R)
Cyclophilin A	NM_008907	CGA GCT CTG AGC ACT GGA GA (F) TGG CGT GTA AAG TCA CCA CC (R)

Green I was incorporated into the PCR buffer provided in the QuantiTech SYBR PCR kit (QIAGEN) to allow for quantitative detection of the PCR product in a 30- $\mu$ l reaction volume. The temperature profile of the reaction was 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 15 s, and annealing and extension at 60 °C for 1 min. Cyclophilin A was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. The size of the PCR product was first verified on a 2.0% agarose gel and then subjected to melting-curve analysis.

## 2.7. Measurement of intracellular ROS

The 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay was used to measure ROS. MC3T3-E1 cells (20,000 per well) were cultured in 96-well plates in  $\alpha$ -MEM with 10% serum. On the second day, cells were serum-starved for 3 h in  $\alpha$ -MEM with 0.2% BSA before TNF $\alpha$  (2 ng/ml) with or without kaempferol and quercetin was added at 10  $\mu$ M concentration. ROS was measured 6 h after addition of TNF $\alpha$  following our optimized protocol [22]. Briefly, cells were washed once with HBSS and incubated in the same buffer containing 5–10  $\mu$ g of DCFH-DA/ml at 37 °C for 30 min. Intracellular fluorescence was detected with excitation at 485 nm and emission at 530 nm using Spectra Max Gemini EM (Molecular Devices, CA).

## 2.8. Statistics

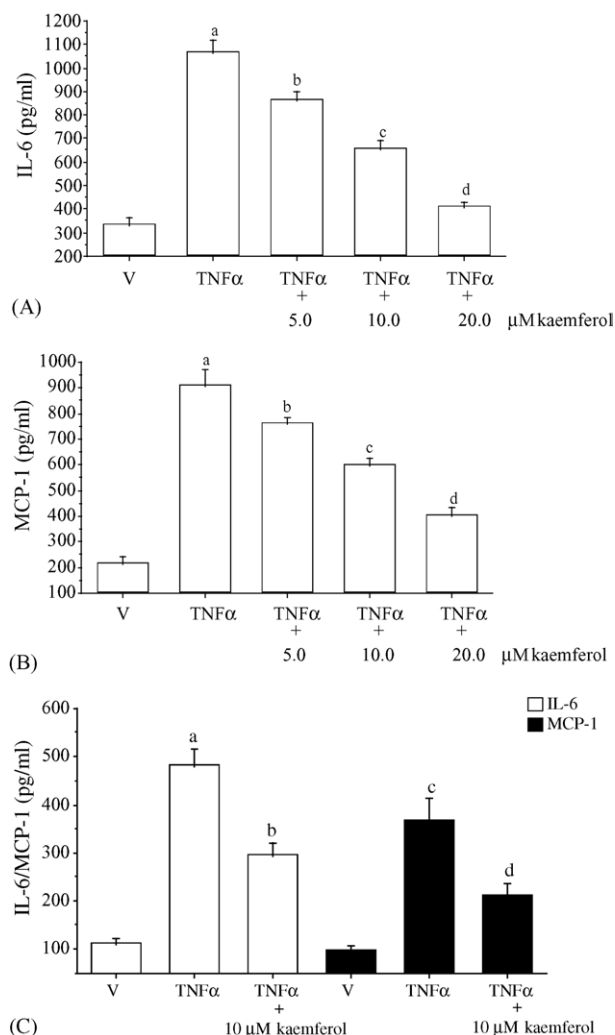
Data are presented as means  $\pm$  S.E. of the indicated number of experiments. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test or Student's *t*-test when appropriate. A *P* value of <0.05 was taken to indicate a statistically significant difference.

## 3. Results

### 3.1. Kaempferol but not quercetin inhibited TNF $\alpha$ -induced secretion of IL-6 and MCP-1 in osteoblasts

TNF $\alpha$  is known to induce synthesis of various osteoclastogenic cytokines from osteoblasts. We first, employed cytokine antibody array to screen for the TNF $\alpha$ -stimulated cytokines that are attenuated by kaempferol in osteoblasts. For screening purposes, we used an established mouse calvarial cell line, MC3T3-E1 cells. Using a mouse cytokine antibody array (containing 32 cytokine antibodies described in Section 2), we found that 20  $\mu$ M kaempferol reduced TNF $\alpha$  (2 ng/ml)-induced secretion of IL-6 and MCP-1 from MC3T3-E1 cells (data not shown). We confirmed this observation by IL-6 and MCP-1 ELISA. We found that kaempferol dose-dependently inhibited TNF $\alpha$ -induced secretion of IL-6 (Fig. 1A) and MCP-1 (Fig. 1B) in MC3T3-E1 cells 18 h after stimulation. This inhibitory effect of kaempferol on TNF $\alpha$ -stimulated IL-6 and MCP-1 production was also observed as early as 6 h (Fig. 1C). Of importance, we also observed that kaempferol alone had no effect in the unstimulated secretion of these cytokines (data not shown).

We confirmed our results in the MC3T3-E1 cell line by studying primary osteoblast cultures. Using these cultures, we showed that kaempferol similarly inhibited TNF $\alpha$ -induced

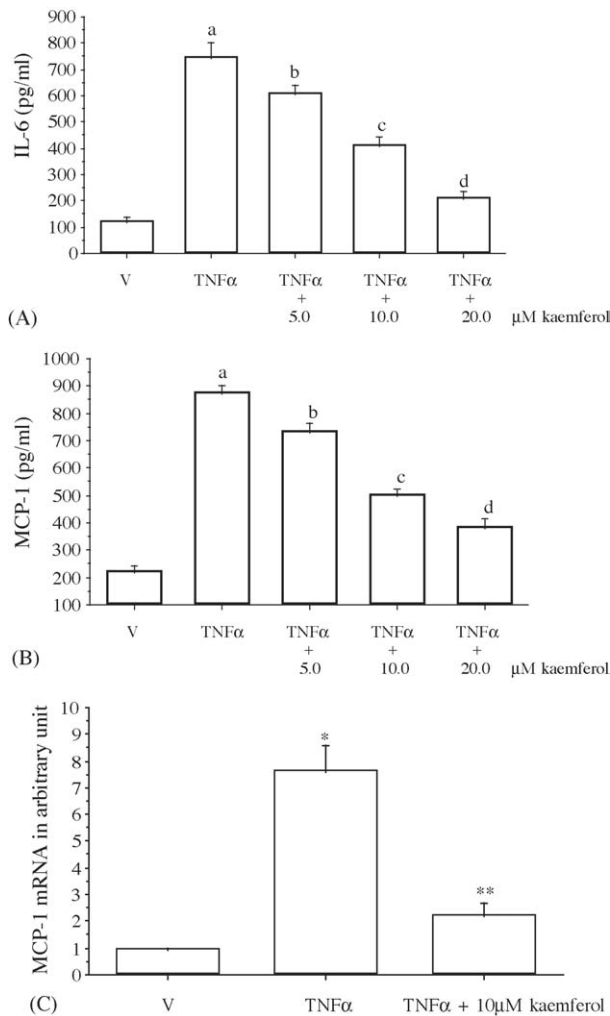


**Fig. 1 – Inhibition of TNF $\alpha$ -stimulated secretion of IL-6 and MCP-1 by kaempferol in MC3T3-E1 cell line. Amount of (A) IL-6 and (B) MCP-1 secreted in the conditioned medium of MC3T3-E1 cells after 18-h incubation with either TNF $\alpha$  or TNF $\alpha$  + 10  $\mu$ M kaempferol at the indicated concentrations: a > b > c > d; *P* < 0.05. Amount of (C) IL-6 [a > b; *P* < 0.05] and (B) MCP-1 [c > d; *P* < 0.05] secreted in the conditioned medium of MC3T3-E1 cells after 6-h incubation with either TNF $\alpha$  or TNF $\alpha$  + 10  $\mu$ M kaempferol at various concentrations indicated. V-vehicle control.**

secretion of IL-6 and MCP-1 secretion with no effect on the unstimulated levels of secretion (Fig. 2A and B). In both MC3T3-E1 and calvarial osteoblasts, quercetin failed to inhibit TNF $\alpha$ -induced secretion of these cytokines, indicating that the effect is specific for kaempferol (data not shown).

### 3.2. Differential inhibition of TNF $\alpha$ -induced secretion of IL-6 and MCP-1 by kaempferol

We examined mRNA levels by QPCR in osteoblasts to assess whether kaempferol inhibits TNF $\alpha$ -induced synthesis of IL-6 and MCP-1 transcripts. Since, the effect of kaempferol in inhibiting TNF $\alpha$ -stimulated secretion of IL-6 and MCP-1 was comparable in MC3T3-E1 cells and primary osteoblasts, we



**Fig. 2 – Inhibition of TNF $\alpha$ -stimulated secretion of IL-6 and MCP-1 by kaempferol in mouse calvarial osteoblasts. (A) Amount of IL-6 and (B) MCP-1 secreted in the conditioned medium of osteoblasts after 18-h incubation with either TNF $\alpha$  or TNF $\alpha$  + kaempferol at various concentrations indicated: a > b > c > d;  $P < 0.05$ . V-vehicle control. (C) Kaempferol inhibits TNF $\alpha$ -stimulated increase of MCP-1 mRNA in MC3T3-E1 cells. Cells were incubated with either vehicle or TNF $\alpha$  or TNF $\alpha$  + 10  $\mu$ M kaempferol for 18 h. QPCR determination of MCP-1 mRNA was performed from 2  $\mu$ g total RNA harvested from the cells. \* > \*\*;  $P < 0.05$ . V-vehicle control.**

performed mRNA determination of these two cytokines in MC3T3-E1 cells. MC3T3-E1 cells were treated with either TNF $\alpha$  or TNF $\alpha$  plus kaempferol for 18 h at concentrations described in Fig. 1A and B. Whereas upregulation of MCP-1 mRNA by TNF $\alpha$  is significantly inhibited by kaempferol (Fig. 2C), there was no change in the expression of IL-6 mRNA (data not shown).

### 3.3. Kaempferol but not quercetin inhibited NF- $\kappa$ B activation by TNF $\alpha$ in osteoblasts

TNF $\alpha$  is known to stimulate NF- $\kappa$ B activation in various cells including osteoblasts. We first studied whether kaempferol or

quercetin exerted any inhibitory effect on TNF $\alpha$  action by disrupting NF- $\kappa$ B activation in osteoblasts. Using mouse primary calvarial osteoblasts, we showed characteristic cytoplasmic and perinuclear localization of the NF- $\kappa$ B p65 subunit labeling as observed in unstimulated osteoblasts (Fig. 3A). Whereas, treating the cells with TNF $\alpha$ , resulted in predominantly intense nuclear labeling of p65 subunit (Fig. 3B). Kaempferol (10  $\mu$ M) robustly inhibited TNF $\alpha$ -stimulated nuclear labeling of p65 subunit (Fig. 3C) whereas the same concentration of quercetin was ineffective (Fig. 3D). Fig. 3E shows quantitative expression of the data on nuclear translocation of p65 subunits under the various conditions described above.

### 3.4. Kaempferol did not inhibit TNF $\alpha$ -induced reactive oxygen species (ROS) production in osteoblasts

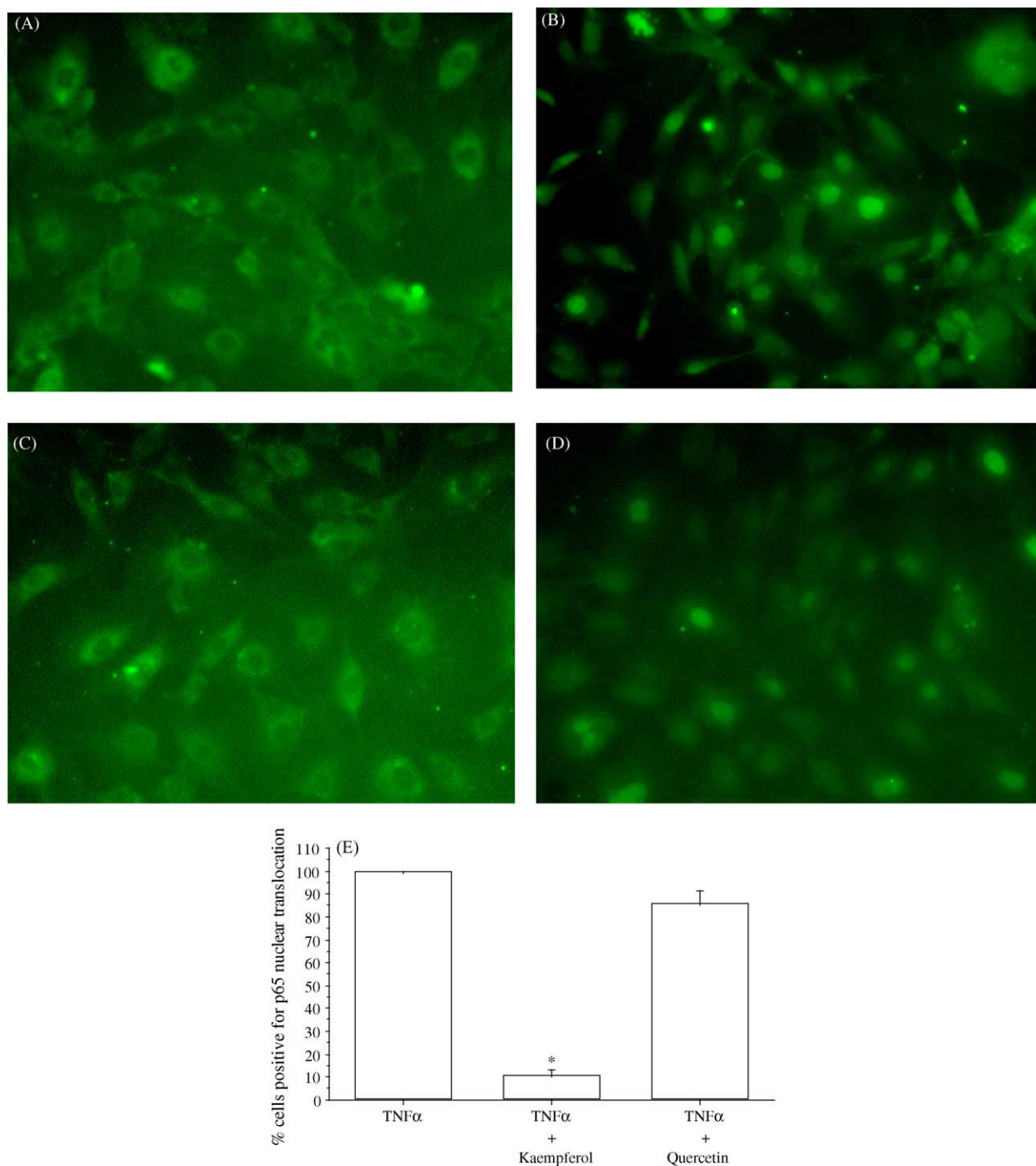
TNF $\alpha$  induces the generation of ROS and thereby participates in cellular signaling by modulating redox-active genes. Stimulation of TNF $\alpha$  receptors rapidly raises the levels of intracellular ROS, which are potent mediators of cellular signaling. We monitored ROS levels in MC3T3-E1 cells using the fluorescent dye H2DCFH-DA [23] and estimated the effect of 10  $\mu$ M kaempferol on ROS production in TNF $\alpha$ -treated cells. TNF $\alpha$  (2 ng/ml) stimulation resulted in a small (~25% over untreated) but significant increase in ROS levels at 6 h (Fig. 4). In cells treated with kaempferol, TNF $\alpha$  continued to stimulate a small (20% over kaempferol-treated) but significant increase in ROS levels at 6 h. Kaempferol treatment alone resulted in lowering of ROS levels (~25% inhibition) (Fig. 4).

### 3.5. Kaempferol and quercetin inhibited RANKL-induced c-fos expression

RANKL, a member of the TNF $\alpha$  family, is secreted by osteoblasts and stimulates osteoclastogenesis. It is well known that c-fos, a transcription factor for AP-1, is required for osteoclast-macrophage lineage determination [24]. Recently, RANKL has been shown to induce c-fos expression in RAW264.7 cells as early as 1 h and remains elevated for more than 6 h [25]. We used this information to study whether kaempferol and quercetin inhibit RANKL-induced c-fos expression in RAW264.7 cells. Fig. 5 shows the concentration-dependent downregulation of RANKL-induced c-fos mRNA expression by kaempferol and quercetin in RAW264.7 cells. Kaempferol had a more pronounced effect than quercetin as inhibition of RANKL-induced c-fos mRNA expression was inhibited by about 15- and 30-fold by 10 and 20  $\mu$ M kaempferol, respectively compared to 8- and 22-fold by quercetin at similar concentrations. These data also suggest that these flavonols exert antiosteoclastogenic effects very early in the process of RANKL-induced osteoclast differentiation.

### 3.6. Kaempferol and quercetin inhibit RANKL-induced osteoclastogenesis

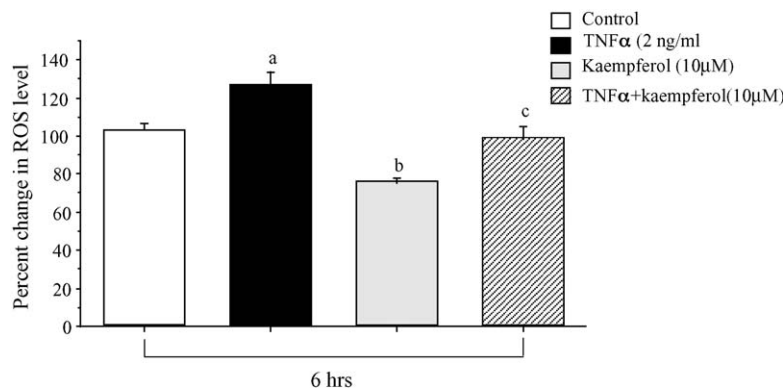
RANKL stimulates osteoclastogenesis with accompanying increases in the expression of osteoclast differentiation



**Fig. 3 – Kaempferol inhibits TNF $\alpha$ -induced NF- $\kappa$ B nuclear translocation.** Mouse primary calvarial osteoblasts were cultured on coverslips. Before the experiments, growth medium was replaced with serum-free medium for 4 h. The cultures were pre-incubated with 10  $\mu$ M kaempferol or quercetin for 10 min, washed with serum-free medium, and stimulated with vehicle (panel A), 2 ng/ml TNF $\alpha$  (panel B), 2 ng/ml TNF $\alpha$  + 10  $\mu$ M kaempferol (panel C), and 2 ng/ml TNF $\alpha$  + 10  $\mu$ M quercetin (panel D) for 1 h. The cultures were then fixed and p65 localization was determined by immunofluorescence. (E) The number of osteoblasts exhibiting nuclear localization of p65 was expressed as a percentage of the total osteoblasts on each coverslip. Data are expressed as mean  $\pm$  S.E.M. of three independent experiments. \* $P < 0.05$ .

genes such as RANK and calcitonin receptor (CTR) [26]. We found that 10  $\mu$ M kaempferol or quercetin significantly inhibited RANKL-induced expression of RANK and CTR mRNAs in RAW264.7 cells (Fig. 6A and B).

Indeed, 10  $\mu$ M kaempferol or quercetin inhibited RANKL-induced formation of TRAP-positive multinucleated (>3 nuclei/cell) cells by ~8.5- and 4.0-fold, respectively (Fig. 6C).



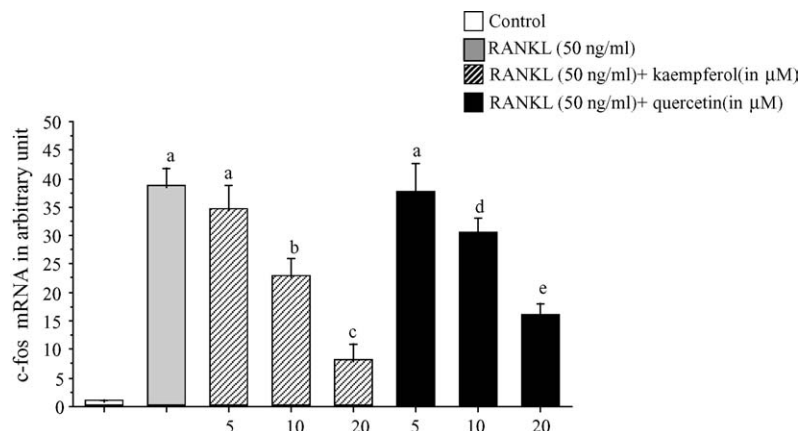
**Fig. 4 – Kaempferol did not inhibit TNF $\alpha$ -induced reactive oxygen species (ROS) production in osteoblasts.** Twenty thousand cells per well were cultured in 96-well plates and exposed to either vehicle or TNF $\alpha$  or TNF $\alpha$  + 10  $\mu$ M kaempferol for 6 and 24 h. The medium was replaced with Hanks' solution containing 5  $\mu$ M H<sub>2</sub>DCFDA, and fluorescence was detected with excitation at 485 nm and emission at 530 nm using Spectra Max Gemini EM: a > c > b;  $P < 0.05$ .

#### 4. Discussion

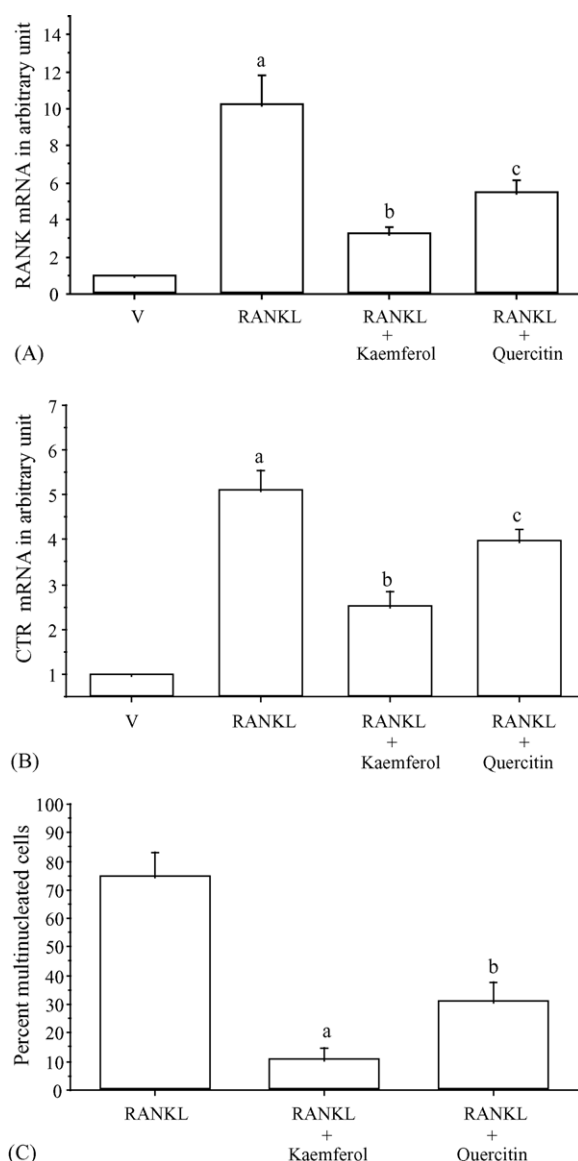
TNF $\alpha$  plays a critical role in the postmenopausal bone loss due to its various permissible actions on osteoclasts [5–11]. Isoflavonoids and flavonols are increasingly recognized for their potential anti-resorptive action attributable, in part, to their capacity to bind the estrogen receptor [17,27,28]. Recently, flavonols such as kaempferol and quercetin have been shown to inhibit bone resorption in rabbit mature osteoclasts and promote osteoclast apoptosis [20]. Our study support and extend these observations for the first time by revealing that kaempferol but not quercetin inhibits TNF $\alpha$ -induced signaling in the osteoblasts, resulting in the reduction of TNF $\alpha$ -induced secretion of IL-6 and MCP-1. Kaempferol and quercetin are classified as phytoestrogens [27]. Our data suggest that kaempferol's action on osteoblast functions studied here appears to be estrogen receptor independent because: (a) quercetin failed to inhibit TNF $\alpha$ -induced NF- $\kappa$ B translocation, and cytokine secretion; (b) prior studies have shown that 17 $\beta$ -estradiol does not inhibit TNF $\alpha$ -induced synthesis of IL-6 in osteoblasts [29,30].

IL-6 and MCP-1 are two potent osteoclastogenic cytokines predominantly derived from osteoblast [31–35]. TNF $\alpha$  is a potent stimulator of MCP-1 and IL-6 secretion from the osteoblasts [13,30,34]. Our data show that kaempferol but not quercetin inhibits TNF $\alpha$ -induced secretion of IL-6 and MCP-1 from osteoblasts. Interestingly kaempferol inhibits TNF $\alpha$ -induced increases of MCP-1 mRNA levels without altering IL-6 mRNA levels. These observations indicate two distinct mechanisms of action of kaempferol in antagonizing TNF $\alpha$  action in osteoblasts.

Our studies investigating the intracellular signaling associated with kaempferol's ability to antagonize TNF $\alpha$  action on osteoblast demonstrate that kaempferol blocks TNF $\alpha$ -induced nuclear translocation of NF- $\kappa$ B. Indeed, MCP-1 transcription has been shown to be regulated by NF- $\kappa$ B [36,37]. We have also investigated the effect of kaempferol on TNF $\alpha$ -induced ROS production. In contrast to our findings in NF- $\kappa$ B translocation, IL-6 and MCP-1 secretion, TNF $\alpha$ -stimulated ROS production was undiminished in the presence of kaempferol. These findings suggest that ROS does not play a role in the inhibitory action of kaempferol on TNF $\alpha$ -stimulated IL-6 and MCP-1 secretion.



**Fig. 5 – Kaempferol more potently than quercetin inhibits RANKL-induced c-fos expression in RAW264.7 monocyte/macrophage cell line.** RAW264.7 cells were treated with either vehicle (V) or 50 ng/ml RANKL or RANKL plus kaempferol or quercetin for 6 h at the indicated concentrations. Inhibition of RANK-induced c-fos mRNA by kaempferol (A) and by quercetin (B) was assessed by QPCR: a > d > b > e > c;  $P < 0.05$ .



**Fig. 6 – Kaempferol more potently than quercetin inhibits RANKL-induced osteoclastogenesis. RAW264.7 cells were treated with either 50 ng/ml RANKL or RANKL + kaempferol/quercetin for 3 days. Ten micrometers of kaempferol or quercetin inhibit RANKL-induced RANK (A) and CTR (B) mRNA expression in RAW264.7 cells assessed by QPCR:  $a > c > b$ ;  $P < 0.05$ . (C) Ten micrometers of kaempferol or quercetin significantly reduce RANKL-induced formation of TRAP-positive multinucleated cells ( $>3$  nuclei/cell).**

Flavonols have been shown to inhibit osteoclastic bone resorption and promote apoptosis of rabbit mature osteoclasts [20]. Our studies complement these findings by demonstrating the effect of flavonols on osteoclast differentiation. We show that both kaempferol and quercetin inhibit RANKL-induced differentiation of RAW264.7 cells to osteoclasts by inhibiting expression of the immediate-early oncogene *c-fos*. To our knowledge, this event represents the most proximal RANKL-induced event in osteoclastogenesis. *c-fos* expression is

indispensable for osteoclastogenesis, as *c-fos* null mice show diminished levels of mature osteoclasts and display osteopetrosis [24,38]. These findings lead us to conclude that kaempferol and quercetin block RANKL-induced osteoclastogenesis by inhibiting *c-fos* expression. Consequently these flavonols were also shown to inhibit RANKL-induced expression of RANK and CTR. Thus, culminating in strongly inhibited RANKL-induced formation of multinucleated giant cells. Of importance to osteoclast differentiation kaempferol appears to be more potent than quercetin in all of the effects studied in RAW264.7 cells.

Our studies reveal the differential action of kaempferol and quercetin on bone cell functions, kaempferol being effective in both osteoblasts and osteoclasts and quercetin being effective only in osteoclasts. The concentration of the flavonols used in our studies (5–20  $\mu$ M) is in the range of plasma concentration of the quercetin derivative, rutin (quercetin-3-*o*-rutoside), i.e.  $\sim 10$   $\mu$ M, that was achieved by feeding rats a semisynthetic diet containing 0.25% rutin that resulted in inhibition of ovariectomy-induced bone loss [19]. Our results provide a possible physiological mechanism to explain the prevention of ovariectomy-induced bone loss by rutin that involves inhibition of RANKL-induced differentiation and osteoclast formation. The  $IC_{50}$  for kaempferol and quercetin were 1.6 and 5.3  $\mu$ M, respectively, for in vitro osteoclastic bone resorption where direct effects of these two compounds were tested [20]. We showed that these two flavonols antagonize the effects of  $TNF\alpha$  and RANKL at concentrations 10- and 25-fold higher than their  $EC_{50}$ , 0.2 and 2 ng/ml, respectively. In particular, the ability of kaempferol to antagonize supraphysiological concentrations of the osteoclastogenic cytokine  $TNF\alpha$  in osteoclasts and RANKL in osteoclasts make it a potential agent for in vivo application, with the promise of better bone health following the insults that result in bone loss.

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