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Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on *in vitro* osteoclastic bone resorption

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Abstract

Several recent studies have suggested that flavonols, a class of phytochemicals with many biological activities, might exert a protective effect against post-menopausal bone loss. In the present study, we investigated the effects of quercetin and kaempferol, two of the major naturally occurring flavonols on the *in vitro* bone resorbing activity of osteoclasts. Our results indicate that both compounds, at concentrations ranging from 0.1 to 100 μM reduce bone resorption in a time and dose-dependent manner. Significant inhibitory effects were observed at concentrations as low as 0.1 μM especially with kaempferol. The IC_{50}s, or concentration inhibitory of 50% of basal resorption, calculated for quercetin and kaempferol were 1.6 and 5.3 μM, respectively. Using highly purified rabbit osteoclasts, we showed that both flavonols directly induce apoptosis of mature osteoclasts in the same dose-range effective for inhibiting bone resorption. When osteoclasts were treated with 50 μM of quercetin and kaempferol, intracellular reactive oxygen species levels decreased significantly by 75 and 25%, respectively, indicating these molecules keep their antioxidant properties at this concentration. However, at concentrations below 50 μM, neither quercetin nor kaempferol exerted antiradical action, suggesting that antioxidant properties cannot fully explain the inhibitory effect on bone resorption. Finally, we report that kaempferol-, but not the quercetin-induced inhibition of bone resorption was partially abolished by the presence of the pure anti-estrogen ICI 182780 suggesting that kaempferol's estrogenic effect could be involved in the inhibition of bone resorption. The present study demonstrates that flavonols widely distributed in human diet such as quercetin and kaempferol, exert a potent inhibitory effect on *in vitro* bone resorption.

Keywords: Osteoclasts; Bone resorption; Apoptosis; Quercetin; Kaempferol; Flavonoids

1. Introduction

During adulthood, bone is continuously remodeled and a balance between resorption of old bone by osteoclasts and formation of new bone by osteoblasts is required for maintenance of skeletal integrity. Osteoclasts are multinucleated cells derived from hematopoietic precursor cells of the monocyte–macrophage lineage [1], and the bone resorption process is closely dependent on the pool size of active osteoclasts [2]. Numerous cytokines, hormones and growth factors control the physiological bone resorption by regulating both the recruitment and differentiation of new osteoclasts or the lifespan of existing osteoclasts by a reduction in the rate of apoptosis [3]. In pathophysiological situations such as post-menopausal osteoporosis, there is an abnormally high bone turnover with enhanced osteoclastic bone resorption. Estrogen deficiency, occurring after the menopause, causes an increase in the number of active osteoclasts which represents the major pathological determinant responsible for the post-menopausal bone loss [3].

A great deal of evidence indicates that osteoporosis is the result of a reciprocal interaction between genetic susceptibility and environmental factors. In this respect, dietary habits might play a fundamental role in the prevention of this disease. Thus, identifying the individual components of the diet that can counteract the typical increase in bone resorption which follows menopause, is of great interest for a non-pharmacological prevention of post-menopausal bone loss.

Several epidemiological studies have demonstrated a lower incidence of osteoporosis in Asian women as

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compared to occidental women [4,5] which is attributable, at least in part, to their higher intake of soyfoods. Indeed, soybeans are particularly rich in isoflavonoids, a subfamily of flavonoids, mainly represented by genistein and daidzein, which significantly prevented bone loss in ovariectomized rats [6,7] and have positive effects on both osteoblast [8,9] and osteoclast [10] activities. Genistein and daidzein share structural similarity with natural estrogen, and their beneficial effects have been attributable to their capacity to bind the estrogen receptor. They are so-called phytoestrogen.

More recent data have reported the potential role of tea drinking [11] and vegetable consumption such as onions [12–14] in protection against osteoporosis, suggesting that some nutrients found in these foods may influence bone remodeling. Tea and onions are not an important source of isoflavones suggesting that isoflavones are not involved in the bone sparing effects of these foodstuffs. However, tea and onions are particularly rich in flavonols, another subfamily of flavonoids, including quercetin and kaempferol. The hypothesis that flavonols may have a positive effect on bone remodeling is strengthened by a recent study [15], which reported that rutin, a glycoside of quercetin, inhibits ovariectomy-induced osteopenia in rats. However, so far, no studies have been carried out to assess the potential effects of flavonols on bone cells. So, in this work, we investigated the effects of quercetin (3,3′,4′,5,7-pentahydroxyflavone) and kaempferol (3,4′,5,7-tetrahydroxyflavone), two of the major dietary flavonols, particularly abundant in fruits, vegetables [16] and beverages such as tea [17], on *in vitro* bone resorption. Our findings demonstrate that these naturally occurring flavonols exert a potent inhibitory effect on bone resorption, and induce apoptosis of the bone resorbing osteoclasts. Despite a very closely related chemical structure, quercetin and kaempferol did not show similar properties: while quercetin exerted only antiradical activity, kaempferol showed, in addition to antioxidant property, a possible interaction with the estrogen receptor.

2. Materials and methods

2.1. Cell culture

Osteoclasts were prepared according to a procedure described previously by Tezuka *et al.* with slight modifications [18]. Unfractionnated bone cells were obtained from long bones of 10-day-old rabbits (Elevage Scientifique des Dombes) sacrificed by immersion in ethanol. Briefly, long bones and scapulae were dissected and minced in αMEM (minimum essential medium Eagle with a alpha modification, Sigma) supplemented with penicillin, streptomycin and glutamine (Sigma). After an agitation by vortexing, the cells were dissociated from bone fragments and collected by centrifugation (4 min, 45 *g*). The cell pellet was resuspended in αMEM supplemented with 10% heat inactivated fetal calf serum (FCS, Dominique Dutscher) and seeded onto culture dishes or plates (Corning). Osteoclasts were characterized by tartrate-resistant acid phosphatase staining, using a TRAP staining kit (Leukocyte acid phosphatase staining kit 387 A, Sigma).

2.2. Purification of osteoclasts

The bone cells were cultured overnight in αMEM with 10% FCS, then washed twice with phosphate-buffered saline (PBS, Sigma), before adding a solution of 0.001% pronase (France-Biochem) in 0.02% ethylene diamine tetraacetic acid solution (EDTA, Sigma). The plates were placed at 37° for 10 min and vortexed twice. The stromal and osteoblastic cells were removed and a highly purified osteoclast population (>99%) was obtained and cultured for 2 hr in a 10% FCS medium, before adding definitive media, containing different concentrations of flavonoids. After purification, 99% of the cells were TRAP+ and only multinucleated cells, with at least 3 nuclei, were considered as osteoclasts. Stock solutions of quercetin and kaempferol (Sigma) were prepared in dimethyl sulfoxide (DMSO, Sigma), such that the final concentrations of the solvent in the cell suspension never exceeded 0.1%. Respective controls were treated with an equal volume of DMSO.

2.3. Assessment of osteoclast apoptosis

Detection of osteoclast apoptosis was by Hoechst staining as described previously [19]. The purified cells were fixed with 3.7% formaldehyde solution in PBS for 10 min, and stained with 0.2 mM Hoechst 33258 (Sigma) for 15 min for visualization of chromatin condensation under a fluorescence microscope (Olympus BH2). Percentages of apoptotic cells were evaluated by calculating the ratio of apoptotic osteoclasts on total number of osteoclasts.

2.4. Assessment of bone resorbing activity

2.4.1. Pit area measurement

The cell suspension was seeded on bovine cortical bone slices (6 mm diameter) placed in each well of a 96-well plate (Corning) for 1 hr at 37° in αMEM supplemented with 10% FCS. Media and non-adherent cells were removed and replaced with fresh media (10% FCS) containing different concentrations of flavonoid. After incubation for 48 hr and removal of the cells, the bone slices were stained with 1% toluidine blue–1% borate for 30 s for assessment by reflected light microscopy. Pit area was measured by using an image analysis system (Biocom) linked to a light microscope (Olympus BH2). When using ICI 182780 (Tocris), cell cultures were pre-incubated in phenol red-free media αMEM (Life Technologies) with the antagonist of estrogen receptors during 2 hr before adding test substances.

2.4.2. Measurement of the pyridinolines cross-links released in culture supernatants by high-performance liquid chromatography (HPLC)

After 48 hr of culture in presence or in absence of either 50 μM of quercetin or kaempferol, the supernatants were harvested and stored at −20° until analysis. The hydroxylysylpiridinoline (HP) content of the supernatants was determined by HPLC as previously described [20]. Briefly, each sample was hydrolyzed with HCl (12 M) for 20 hr at 105°, then extracted using F1 cellulose column (Biorad laboratory). HP cross-links were eluted with distilled water, freeze-dried overnight, then the residues were dissolved in 1% heptafluorobutyric acid and separated on a C18 reversed phase column. HP was detected by measuring natural fluorescence using a Jasco FP1520 spectrofluorimeter (Merck) with excitation $\lambda = 297$ nm and emission $\lambda = 380$ nm. The amount of HP in the supernatant was calculated in nano molar per well and expressed as percent of control.

2.5. Quantification of intracellular peroxides by DCF assay

2,7-Dichlorofluorescein (DCF) assay for the quantification of intracellular reactive oxygen species (ROS) was used as previously described [21]. Briefly, 2,7-dichlorofluorescein diacetate is a non-polar, non-fluorescent and stable compound that can readily diffuse into the cell membrane. Within the cells, cytosolic enzymes cleave the acetate and generate an hydrolyzed compound, 2,7-dichlorofluorescein. In the presence of peroxides, 2,7-dichlorofluorescein is oxidized to the highly fluorescent form of DCF. Purified osteoclasts cultured for 4 hr were washed once with PBS and incubated with 1 μM 2,7-dichlorofluorescein diacetate (Sigma) at 37° for 15 min. After three washings with PBS, the cells were lysed in 1 mL of H_2O and the fluorescence was measured using a spectrofluorometer (Shimatzu) at $\lambda_{ex} = 503$ and $\lambda_{em} = 529$ nm. The results were calculated in arbitrary unit of fluorescence per milligram of protein and expressed as percent of control.

2.6. Statistical analysis

All data are expressed as mean ± SEM and statistical significance was determined by Mann–Whitney's test using Statview® software. A value of $P < 0.05$ was considered as significant.

3. Results

3.1. Effects of quercetin and kaempferol on bone resorption

To test the effects of quercetin and kaempferol on bone resorption, osteoclasts, the only known cells in charge of bone resorption, were prepared from rabbit long bones. This culture system yielded mature multinucleated osteoclasts mixed with other cells such as mononucleated osteoclasts, osteoblasts and stromal cells. When these unfractionated rabbit bone cells were cultured on bone slices for 48 hr in presence of several concentrations (0.1–100 μM) of each flavonoid, bone resorption evaluated by pit area measurement was reduced in a dose-dependent manner (Fig. 1A). Kaempferol exerted significant inhibitory effects on bone resorption at a concentration as low as 0.1 μM. The inhibition concentration IC_{50} measured for kaempferol and quercetin was 1.6 and 5.3 μM, respectively.

The inhibition of bone resorption obtained by pit area measurement was confirmed by using a biochemical evaluation of bone resorption based on HPLC measurement of HP, a collagen cross-link molecule released in culture supernatants during bone resorption. As shown in Fig. 1B, when osteoclasts cells were treated with 50 μM of either flavonol, a highly significant decrease in bone resorption was obtained by HP measurement.

3.2. Effects of quercetin and kaempferol on osteoclast apoptosis

We then investigated whether quercetin and kaempferol can modulate osteoclast apoptosis. For this purpose, rabbit osteoclasts were purified from unfractionated bone cells using a method which led a very high cell purity [22]. Osteoclasts displaying characteristics of apoptosis as chromatin condensation and DNA-fragmentation can be easily distinguished from normal cells by using Hoechst staining and detection with fluorescence microscopy (Fig. 2A and B). As shown in Fig. 2C, quercetin and kaempferol increased osteoclastic programmed cell death in a dose-dependent manner. After 48 hr of culture, the number of apoptotic osteoclasts in cultures treated with 50 μM of each flavonol was increased approximately 3-fold as compared to control culture. At lower doses (0.1 and 1 μM), the kaempferol effect was more pronounced than that of quercetin. A time course study of apoptotic changes in osteoclasts cultured in the presence or absence of flavonoid (50 μM) is shown in Fig. 2D. The proapoptotic effect with quercetin occurred more rapidly than with kaempferol. Significant differences as compared to control culture were observed as early as 12 hr of culture with quercetin and only after 48 hr with kaempferol.

3.3. Inhibition of reactive oxygen species production

Osteoclasts are cells known to produce a lot of ROS, especially H_2O_2, and these ROS have been reported to be involved in normal osteoclastic bone resorption [23] as well as in increased bone resorption [24]. We tested the antioxidant effects of quercetin and kaempferol in osteoclasts, by measuring intracellular ROS production. As reported in Fig. 3, quercetin at a concentration of 50 μM

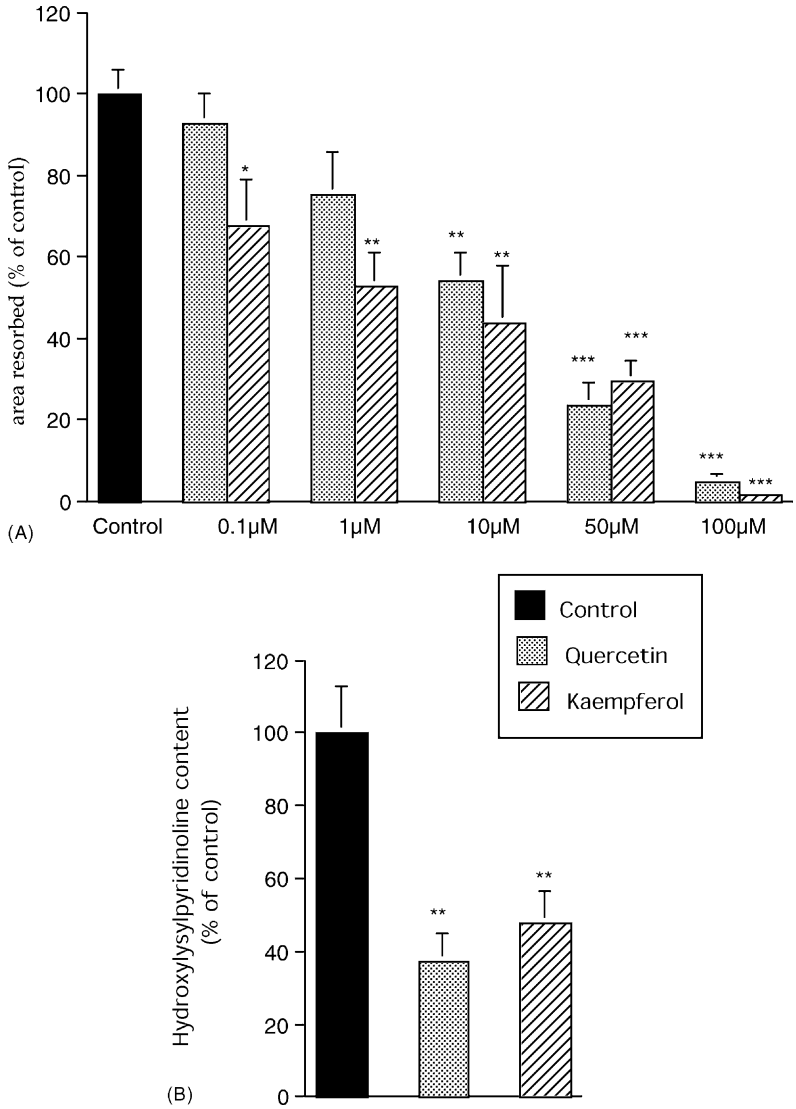

Fig. 1. Effect of different concentrations of quercetin and kaempferol on osteoclastic bone resorption. Osteoclasts were cultured on cortical bovine slices during 48 hr in media containing either vehicle (0.1% DMSO = control) or either flavonols (0.1–100 µM) and bone resorption was assessed by measurement of total area of resorption pits (A). Inhibition of bone resorption was confirmed by HP measurement in supernatants of cultures treated with 50 µM of quercetin and kaempferol (B). Results are expressed as percent of control. Values are mean ± SEM of three independent experiments (N = 5 for pit area measurement); (∗) $P < 0.05$, (∗∗) $P < 0.01$ and (∗∗∗) $P < 0.001$ compared with control group.

reduced significantly ROS level by 75%. At the same concentration, kaempferol also showed antioxidant property but to a lesser extent namely by 35%. At concentrations lower than 50 µM, neither quercetin nor kaempferol showed any significant effect in decreasing ROS production. However, it can be noticed that both compounds have prooxidant properties at lower doses, particularly for kaempferol.

3.4. ER-mediated effects of flavonoids on bone resorption

Using ICI 182780, a pure antagonist which can block estrogen receptor (ER), we examined whether quercetin and kaempferol effects on bone resorption were ER mediated or not. ICI at 10^{-7} M did not modulate bone resorption assessed by pit area measurement (Fig. 4). When combined with kaempferol (50 µM), ICI partially reversed the inhibitory effect on bone resorption, indicating that kaempferol activity on osteoclasts could be mediated, at least in part, by estrogen receptors. In contrast, when ICI was added to the culture in presence of quercetin, the inhibitory effect on bone resorption was not significantly reversed.

4. Discussion

Recent epidemiological and experimental data have suggested that several foodstuffs regularly consumed by humans could have an interest in prevention of bone loss

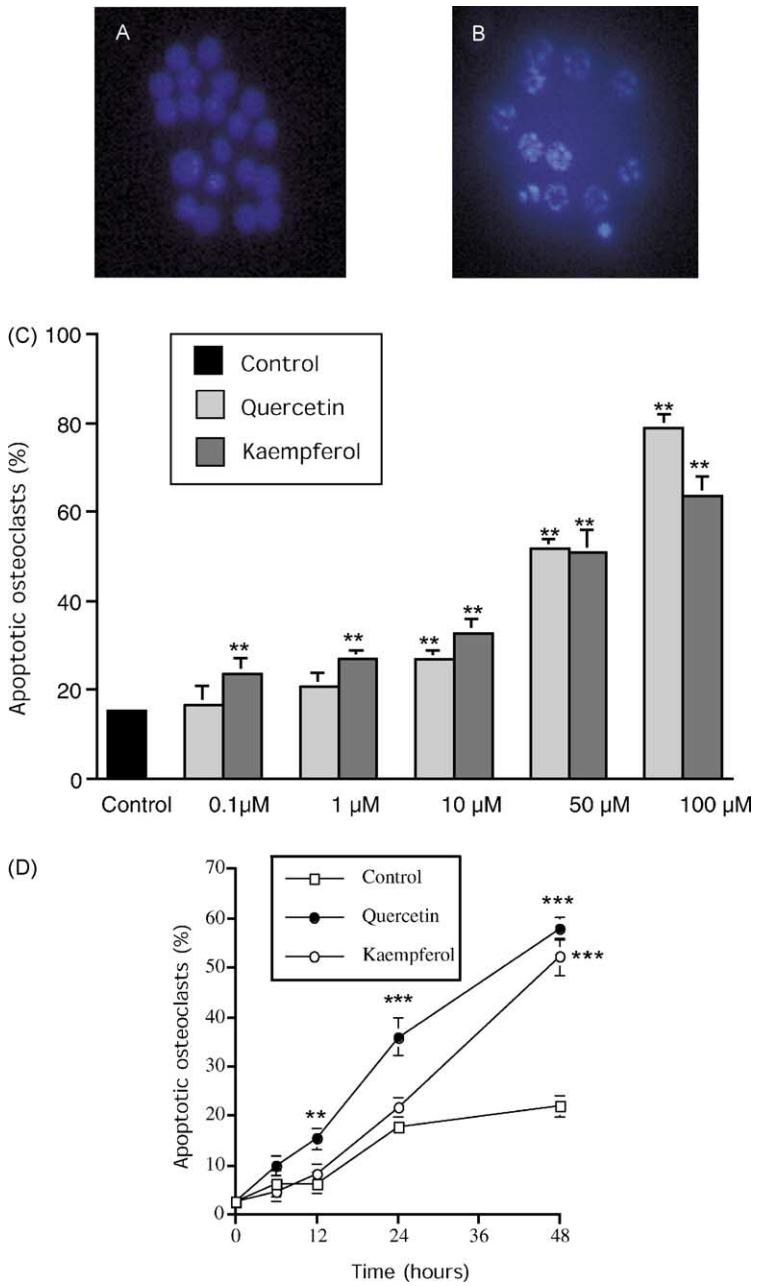

Fig. 2. Dose- and time-dependent effect of quercetin- and kaempferol-induced osteoclast apoptosis. Pure rabbit osteoclasts were cultured for 48 hr in αMEM containing either vehicle (control) or either flavonol (0.1–100 μM). Apoptosis was detected by staining cells with 0.2 mM Hoechst 33258 to visualize chromatin condensation. (A) and (B) A fluorescence micrograph (magnification, 400×) of normal and apoptotic osteoclast, respectively. (C) After quantification a dose-dependent effect of quercetin and kaempferol on osteoclast apoptosis. (D) A time course study of apoptotic changes induced by 50 μM of quercetin and kaempferol. Results are expressed as percentage of apoptotic osteoclasts and values represent the mean ± SEM of two independent experiments (N = 6 for each); (**) $P < 0.01$ and (***) $P < 0.001$ compared with control group.

occurring at the menopause. Muhlbauer *et al.* [14] have recently shown that several vegetables can significantly inhibit bone resorption in the rat, the strongest effect being obtained with onion extracts. Hegarty *et al.* [11] recently reported among elderly women in Great Britain that tea drinkers had significantly greater bone mineral density as compared to non-tea drinkers, suggesting that tea consumption may protect against osteoporosis. Possible candidates as bioactive molecules present in onion and tea are flavonols. This issue has been recently addressed by Horcajada-Molteni *et al.* [15] who demonstrated that rutin, a quercetin glycoside, can preserve bone mass in ovariectomized rats by slowing down *in vivo* bone resorption. In this study we extend these results by focusing on the potential effects of quercetin and kaempferol, two of the main naturally occurring flavonols, on the bone resorbing activity of osteoclast cells. By using two distinct methods, our results have shown that both compounds reduced

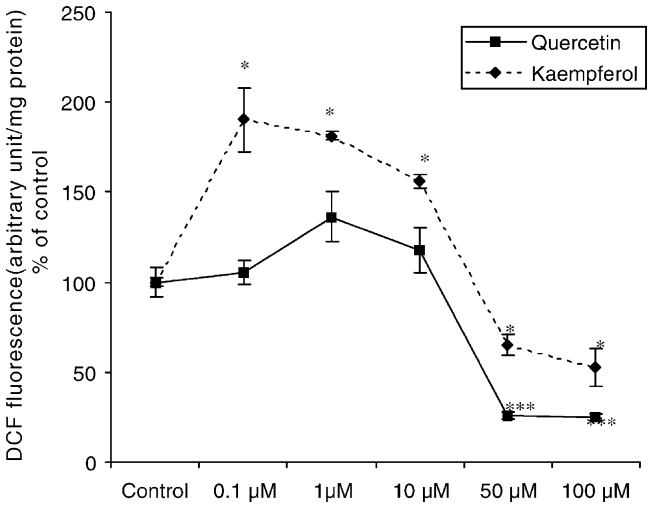

Fig. 3. Dose-effect of quercetin and kaempferol on intracellular ROS production in osteoclasts measured via DCF fluorescence. Results were calculated as arbitrary unit of fluorescence per milligram of protein and expressed as percent of control. Each point represents mean ± SEM (N = 6); (∗) $P < 0.05$ and (∗∗∗) $P < 0.001$ compared with control group.

dramatically the *in vitro* bone resorption. In the case of kaempferol, significant effects were obtained at very low concentration (0.1 μM) suggesting that its effects are more potent than quercetin. For both flavonols, the IC_{50} values are not far from the plasma concentrations, which are in the micromolar range [25].

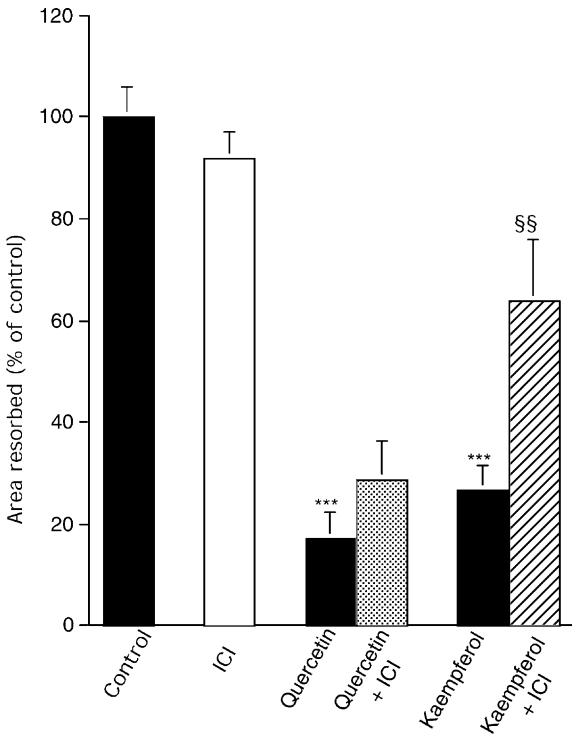

Fig. 4. Effect of ICI 182780, a pure antagonist of estrogen receptor, on the quercetin- and kaempferol-induced inhibition of bone resorption. ICI at 10^{-7} M was added to bone cells culture in the presence or absence of 50 μM of both flavonols; (∗∗∗) $P < 0.001$ vs. control, (§§) $P < 0.01$ vs. kaempferol alone.

It is now clearly established that osteoclasts are cells with a short lifespan and they die rapidly by apoptosis or programmed cell death [26], a process common to many regenerating tissues. Estrogens [27,28] and biphosphonates [29], which are the more potent antiresorptive agents currently used in the treatment of post-menopausal osteoporosis, act at least in part by inducing osteoclast apoptosis. This means that control of osteoclast lifespan may represent a key step in regulation of bone resorption. We have shown that quercetin and kaempferol directly promoted the spontaneously occurring osteoclast apoptosis in a dose range which correlated well with those for inhibition of bone resorption. Kaempferol-induced stimulation of apoptosis occurred at lower concentrations than quercetin, but quercetin effects were observed earlier, indicating they could act by distinct mechanisms. Proapoptotic effects of quercetin have been well documented in other cell systems, especially in malignant cell lines [30,31]. In osteoclasts, the flavonol-induced stimulation of apoptosis could explain, at least partly, the inhibition of bone resorption.

Flavonoids have potent antioxidant properties due to their ability to scavenge ROS [32], probably related to the phenolic hydroxyl groups attached to the ring structures. Osteoclasts express NADPH oxydase, a superoxide-generating enzyme [33] and ROS are involved in increased osteoclastic bone resorption [23,24]. By using 2′,7′-dichlorofluorescein diacetate (DCF-DA) a well-known fluorescent probe of ROS generation, we have shown that quercetin and kaempferol at a concentration of 50 μM lowered ROS in our cell system, indicating that these molecules keep their antioxidant properties at this concentration. The quercetin antioxidant activity was largely more efficient than kaempferol. From a chemical structure point of view, the two compounds differ only by the presence of an additional free hydroxyl at C3′ in quercetin structure (Fig. 5). This additional 3′-OH group produces the quercetin configuration with the 3′,4′-dihydroxystructure which is responsible for an increase in antioxidant activity, as recently reported [34]. For both compounds, the ability to reduce osteoclast ROS production was not observed at concentrations below 50 μM, although at lower concentrations they markedly inhibited bone resorption and increased osteoclast apoptosis, especially in the case of kaempferol. At these concentrations, rather an antioxidant effect, it is a prooxidant effect which is observed, prooxidant effect which has yet been reported for quercetin [35]. Therefore, the antioxidant properties of flavonols cannot fully explain the mechanism whereby quercetin and kaempferol exert their inhibitory effect on bone resorption and their proapototic effect on osteoclast. Another possibility would be that quercetin or kaempferol which share structural similarity with 17β-estradiol (Fig. 5) acts through an ER, as genistein and daidzein do. ERs exist as two subtypes ERα and ERβ and recent studies have reported that osteoclasts could express both receptors [36,37]. To clarify the possible binding interaction between

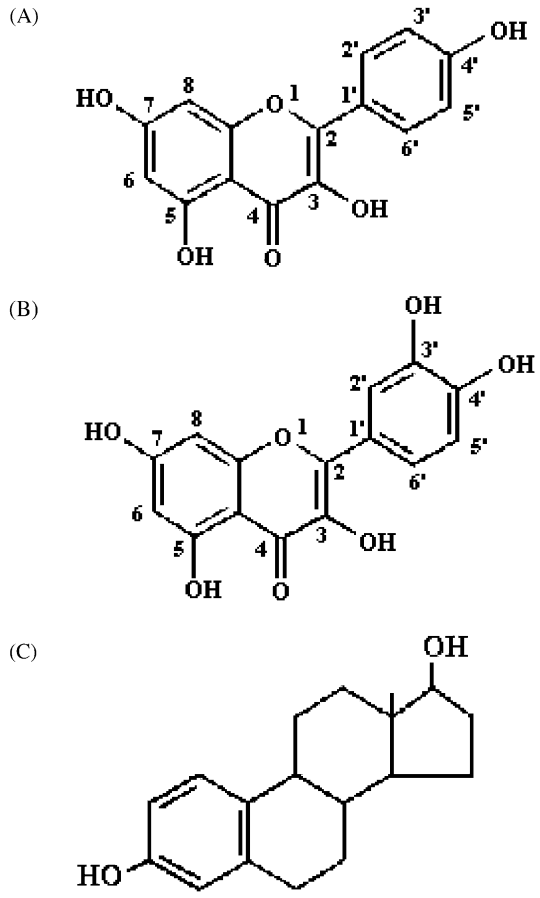

Fig. 5. Chemical structures of kaempferol (3,4′,5,7-tetrahydroxyflavone) (A), quercetin (3,3′,4′,5,7-pentahydroxyflavone) (B), and 17β-estradiol (C).

quercetin and kaempferol with ER, we examined the effect of ICI 182780 on the flavonol-induced inhibition of bone resorption. Only the inhibitory effects of kaempferol on bone resorption, but not those of quercetin, were significantly reversed by ICI used at the concentration of 10^{-7} M suggesting that kaempferol estrogenic properties would be more potent than quercetin. The discrepancy between quercetin and kaempferol interaction with ER could be due to difference in their chemical structure. Indeed, Kuiper *et al.* [38], who reported ranking of estrogenic potency of phytoestrogen for ER subtypes, showed that potency of kaempferol was largely greater than quercetin, which is in accordance with our results. Taken together, it is possible to speculate that kaempferol is more efficient than quercetin in inhibition of bone resorption by a mechanism involving ER. However, others mechanisms of action for quercetin and kaempferol can be evocated, such as inhibition of activation of two key transcription factors NFκB and AP-1 involved in differentiation, survival and activation of osteoclasts [39,40]. Indeed, quercetin has the ability to attenuate NFκB [41] and AP-1 [42] activation.

In conclusion, the data presented in this study demonstrate that quercetin and kaempferol, two natural flavonols widely distributed in the human diet, inhibit the bone resorbing activity of osteoclasts. Although the mode of action remains to be clarified, our findings provide a possible explanation for the protective effect of high intake of flavonols in post-menopausal bone loss. Further research in this field would elucidate the molecular mechanisms involved in the flavonols-induced inhibition of bone resorption and would strengthen a new approach based on non-pharmacological management of osteoporosis.

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