Polymethoxy Flavonoids, Nobiletin and Tangeretin, Prevent Lipopolysaccharide-Induced Inflammatory Bone Loss in an Experimental Model for Periodontitis

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Abstract. Nobiletin, a polymethoxy flavonoid (PMF), inhibits systemic bone resorption and maintains bone mass in estrogen-deficient ovariectomized mice. This study examined the anti-inflammatory effects of PMFs, nobiletin, and tangeretin on lipopolysaccharide (LPS)-induced bone resorption. Nobiletin and tangeretin suppressed LPS-induced osteoclast formation and bone resorption and suppressed the receptor activator of NFκB ligand–induced osteoclastogenesis in RAW264.7 macrophages. Nobiletin clearly restored the alveolar bone mass in a mouse experimental model for periodontitis by inhibiting LPS-induced bone resorption. PMFs may therefore provide a new therapeutic approach for periodontal bone loss.

Keywords: polymethoxy flavonoid, inflammatory bone resorption, periodontal disease

Nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) and tangeretin (5,6,7,8,4′-pentamethoxy flavone) are polymethoxy flavonoids (PMFs) abundantly present in oranges peel, and they exhibit several biological activities. Nobiletin has an anti-proliferative effect on various types of cancer cells (1), attenuates ovalbumin-induced eosinophilic airway inflammation in asthmatic rats (2), and Type II collagen–induced arthritis (3). Both nobiletin and tangeretin regulate adipocyte differentiation and adipocytokine secretion (4). Nobiletin markedly suppresses bone resorption and restores the bone loss in ovariectomized (OVX) mice, a model of postmenopausal osteoporosis (5).

Bone remodeling is regulated by osteoclastic bone resorption and new bone formation by osteoblasts. Prostaglandin E2 (PGE2) is mainly produced by osteoblasts and it acts as a potent stimulator of bone resorption associated with inflammation (6). Nobiletin suppresses the interleukin (IL)-1-induced activation of NFκB-dependent transcription, cyclooxygenase (COX)-2 expression, and PGE2 production in mouse osteoblasts (5). Therefore, PMFs may exert beneficial effects on PGE-related inflammatory bone diseases.

Periodontitis is a bone destructive disease accompanied with inflammation. Lipopolysaccharide (LPS) is a known pathogen associated with periodontitis, an infectious disease of mixed Gram-negative bacteria that is associated with bone resorption. A previous study established an original model for mouse periodontitis associated with bone resorption in alveolar bone induced by LPS injection into the lower gingiva and successfully detected LPS-induced alveolar bone loss (7). This model of periodontitis demonstrated that LPS induces the loss of alveolar bone in wild-type, but not in membrane-bound PGE synthase-1 (mPGES-1)-deficient mice, suggesting that mPGES-1-dependent PGE production is essential for LPS-induced periodontal bone resorption (7). The present study examined the effects of PMFs (nobiletin and tangeretin) on LPS-induced bone resorption in vitro and on alveolar bone mass in the mouse model for periodontitis.

Nobiletin and tangeretin (Fig. 1A) were obtained from Wako Co., Ltd., Tokyo. Newborn, 5-day-old, and 6-week-old mice of the ddy strain were obtained from Japan SLC (Hamamatsu). Primary osteoblastic cells were isolated from newborn mouse calvariae. Mouse bone marrow cells were co-cultured with primary osteoblasts...
Fig. 1. Effects of nobiletin and tangeretin on LPS-induced osteoclast formation and PGE2 production in co-cultures of osteoblasts and bone marrow cells and on the bone-resorbing activity induced by LPS in mouse calvarial organ cultures. A) Chemical structure of nobiletin and tangeretin. B) Mouse bone marrow cells and osteoblastic cells were co-cultured for 7 days with 30 μM nobiletin or 30 μM tangeretin in the presence of LPS (1 ng/ml). The cells were stained for TRAP, a specific marker for osteoclasts, and the number of TRAP-positive multinucleated cells containing 3 or more nuclei was counted. Bar = 1 mm. C) The level of PGE2 was measured by EIA using the conditioned medium of the co-cultures. D) RAW264.7 cells, murine macrophages, were cultured for 5 days with nobiletin (30 μM) or tangeretin (30 μM) in the presence of sRANKL (100 ng/ml). The number of TRAP-positive multinucleated cells containing 3 or more nuclei was counted. Bar = 1 mm. E) Mouse calvariae were dissected in half and then were cultured with or without nobiletin (30 μM) or tangeretin (30 μM) in the presence of LPS (10 μg/ml) for 5 days. The concentration of calcium in the medium was measured to calculate the bone-resorbing activity. Data are expressed as the mean ± S.E.M. of 3 – 5 independent wells. A significant difference between the two groups is indicated, **P < 0.01 vs. control, ##P < 0.01 vs. LPS.
for 7 days, and the cells were stained for tartrate-resistant acid phosphatase (TRAP). RAW264.7 murine macrophages were cultured and treated with soluble type of receptor activator of NF-κB ligand (RANKL) to detect osteoclastogenesis, since RANKL has been shown to participate in osteoclastogenesis (3). TRAP-positive multinucleated cells were counted as osteoclasts. Mouse calvariae were collected from 5-day-old mice and then were cultured for 5 days. The bone-resorbing activity was determined by measuring the concentration of calcium in the conditioned medium (8).

Mandibular alveolar bone was collected from the mouse lower gingiva under a microscope and then was cultured for 24 h in BGJb containing 1 mg/ml BSA. The alveolar bone was transferred to a new medium, with or without LPS, and was cultured for 5 days. The bone-resorbing activity was determined by measuring the concentration of calcium in the conditioned medium (7). LPS (25 μg) was dissolved in 50 μl of PBS and injected into the outside of the mouse lower gingiva on days 0, 2, and 4. PBS was injected into the lower gingiva at each time point as a control. The mandibular alveolar bone was collected 7 days after the first injection (7). The bone mineral density (BMD) of the mandibular alveolar bone was measured by dual X-ray absorptiometry (model DCS-600R; Aloka, Tokyo). The BMD was calculated by dividing the bone mineral content of the measured area by the area. All procedures were performed in accordance with the institutional guidelines and permission for animal research. Any significance of differences was analyzed by Dunnett’s method.

LPS markedly induced osteoclast differentiation in co-cultures of mouse bone marrow cells and osteoblasts on day 7, while 30 μM nobiletin or tangeretin clearly suppressed the LPS induced osteoclast formation (Fig. 1B). The effects of nobiletin and tangeretin on osteoclast formation were concentration-dependent in the range of 3 – 30 μM in the co-cultures (data not shown). The level of PGE2 in the conditioned medium treated with LPS was higher than that of the control, and was completely suppressed by adding nobiletin or tangeretin (Fig. 1C). RAW264.7 macrophages differentiate into osteoclasts in response to soluble RANKL (sRANKL). The addition of 30 μM nobiletin or tangeretin significantly suppressed the sRANKL-induced osteoclastogenesis in RAW264.7 cells (Fig. 1D), thus indicating that PMFs act on osteoclast precursors and suppress the differentiation into osteoclasts. The effects of nobiletin and tangeretin on bone resorption in mouse calvarial organ cultures were examined by the increase in calcium in the conditioned medium. LPS markedly induced bone-resorbing activity in calvarial cultures. Both nobiletin and tangeretin (30 μM) significantly suppressed the bone-resorbing activity induced by LPS (Fig. 1E), thus indicating that PMFs clearly suppress the bone resorption associated with inflammation in bone tissues.

Periodontal disease, such as periodontitis, is a typical bone disease accompanying inflammation and loss of alveolar bone. A new experimental model for periodontitis has been established in vitro and in vivo (7). This model was used to examine the effects of PMFs (nobiletin and tangeretin) on the pathogenesis of periodontitis. Mandibular alveolar bone was collected from the mouse lower mandible (Fig. 2A) and then was cultured with or without LPS to detect bone resorption measured by calcium in the medium. There was an increase in bone-resorbing activity in LPS-treated alveolar bone in vitro,

![Fig. 2. Effects of nobiletin and tangeretin on LPS-induced bone loss of mouse mandibular alveolar bone in vitro. A) Mandibular alveolar bone was collected from mouse lower gingiva under a microscope. B) The collected mandibular alveolar bone was cultured for 24 h in BGJb containing 1 mg/ml BSA. The alveolar bone was transferred to a new medium and then was cultured for 5 days with or without LPS (10 μg/ml). Nobiletin (30 μM) and tangeretin (30 μM) were added to the cultures in the presence of LPS (10 μg/ml). The bone-resorbing activity was measured by the amount of calcium in the medium. Data are expressed as the mean ± S.E.M. of 5 independent wells. A significant difference between the two groups is indicated, **P < 0.01 vs. control, ##P < 0.01 vs. LPS.](image-url)
and both nobiletin and tangeretin completely suppressed the bone resorption induced by LPS in mandibular alveolar bone (Fig. 2B). LPS was injected into the gingiva in the lower mandible, and the alveolar bone was collected from the mice on day 7 for the measurement of BMD by dual X-ray absorptiometry. LPS administration induced a significant decrease in BMD of mandibular alveolar bone in mice (Fig. 3). The injection of PBS, as a control, did not affect the alveolar BMD. The simultaneous administration of nobiletin (300 μg/mouse) with LPS significantly restored the LPS-induced loss of the alveolar bone mass in vivo (Fig. 3).

The present study showed that LPS-induced bone resorption in the calvaria and alveolar bone was clearly suppressed by PMFs, nobiletin, and tangeretin. Both nobiletin and tangeretin suppressed osteoclast formation and PGE$_2$ production in co-cultures of bone marrow cells and osteoblasts. The bone-resorbing factors associated with inflammation, such as IL-1, LPS, and TNF, induce PGE$_2$ production by osteoblasts and the RANKL expression on their surface (9). Two-types of cyclooxygenase, COX-1 and COX-2, are expressed in osteoblasts, and COX-2 is markedly induced by these inflammatory factors to elicit PGE$_2$ production by osteoblasts (9, 10).

![Figure 3](image-url) Administration of nobiletin restored LPS-induced bone loss of mandibular alveolar bone in the mouse. As a model for experimental periodontitis, LPS (25 μg/mouse) was injected into the mouse lower gingiva on days 0, 2, and 4. As a control, PBS was injected into the lower gingiva at each time point. Nobiletin (30, 100, and 300 μg/mouse; open circles) and tangeretin (30, 100, and 500 μg/mouse; closed circles) were injected into the mouse lower gingiva with LPS in some animals. The mandibular alveolar bone was collected at 7 days after the first injection, and BMD of the respective mandibular alveolar bone was measured. The ranges of the mandibular alveolar BMD in control mice (28.87 ± 0.35) and LPS-injected mice (26.18 ± 0.39), $P < 0.01$ vs. control, are shown as shaded rods, respectively. Data are expressed as the mean ± S.E.M. of 5 – 8 mice. A significant difference between the two groups is indicated, *$P < 0.05$ vs. LPS.

Nobiletin suppresses the IL-1-induced activation of NFκB-dependent transcription, COX-2 expression, and PGE$_2$ production in mouse osteoblasts, and nobiletin suppresses IL-1-induced osteoclastogenesis (5). Murakami et al. (11) reported that nobiletin suppresses LPS-induced COX-2 expression in murine macrophages. Furthermore, the present study showed that PMFs suppress osteoclastogenesis in RAW264.7 macrophages (Fig. 1D), thus indicating the direct action of PMFs in osteoclast precursors. Therefore, PMFs may suppress inflammatory bone resorption not only by a PGE$_2$-dependent mechanism, but also by the direct action in osteoclast precursors.

Periodontitis is a destructive bone disease accompanied with inflammation. A new model was developed for mouse periodontitis associated with bone resorption using LPS in alveolar bone (7). Treatment with indomethacin suppresses LPS-induced loss of alveolar bone in mice (C. Miyaura, unpublished data), and mPGES-1-deficient mice are resistant to LPS-induced bone loss (7). These data suggest the production of PGE$_2$ to thus be responsible for LPS-induced alveolar bone loss. The present study showed that PMFs suppressed LPS-induced bone resorption of alveolar bone in the model for mouse periodontitis. Therefore, PMFs may protect against the loss of mandibular alveolar bone in periodontal diseases. Pharmaceutical studies are needed to develop PMF-specific solvent materials for dental ointment to promote the clinical application of PMFs for patients with periodontitis.

Natural ingredients including various flavonoids may act as a beneficial factor for bone tissues and prevent the lifestyle-related diseases including osteoporosis and osteoarthritis. Genistein, a soybean isoflavone, prevents bone loss in OVX mice, a model for osteoporosis (12). Hesperidin, a citrus flavonoid, has also been shown to attenuate bone loss in OVX mice (13). Nobiletin prevents systemic bone loss in OVX mice (5). These data suggest that the intake of the flavonoids may contribute to the prevention of postmenopausal osteoporosis. The present study clearly showed that citrus-derived PMFs exhibit anti-inflammatory effects on a model for periodontitis to maintain the mandibular alveolar bone mass. Further studies are needed to confirm the possible clinical application of PMFs for the treatment of periodontal diseases.

**References**


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