Inhibitory Effects of Bee Pollen *Cistus ladaniferus* Extract on Bone Resorption in Femoral Tissues and Osteoclast-Like Cell Formation in Bone Marrow Cells *in Vitro*

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The effects of bee pollen extract on osteoclastic bone resorption *in vitro* were investigated. The water-solubilized extracts were obtained from the bee pollen of *Cistus ladaniferus*. Femoral-diaphyseal or -metaphyseal tissues of rats were cultured for 48 hr in medium containing either vehicle, bone-resorbing factor, or bone-resorbing factor plus bee pollen extracts (10, 100, or 1000 μg/ml of medium). Culture with parathyroid hormone [human (1-34) PTH; 10^{-7} M], prostaglandin E_2 (PGE_2; 10^{-5} M), or 1,25-dihydroxyvitamin D_3 (10^{-6} M) caused a significant decrease in calcium content in the diaphyseal or metaphyseal tissues. These decreases were completely prevented in the presence of bee pollen extracts (10, 100, or 1000 μg/ml). The presence of PTH (10^{-7} M) caused a significant increase in medium glucose consumption and lactic acid production in the diaphyseal or metaphyseal tissues. These increases were significantly inhibited by culture with bee pollen extracts (10, 100, or 1000 μg/ml). Mouse bone marrow cells were cultured for 7 days in the presence of bone-resorbing factor *in vitro*. Culture with PTH (10^{-7} M), PGE_2 (10^{-5} M), tumor necrosis factor-α (10 ng/ml of medium), or lypopolysaccharide (10 μg/ml of medium) caused a significant increase in osteoclast-like cell formation. These increases were significantly inhibited in the presence of bee pollen extracts (10, 50, or 100 μg/ml of medium). This study demonstrates that bee pollen extract has inhibitory effects on osteoclastic bone resorption *in vitro*.

Key words —— bone resorption, osteoclastic cell formation, bee pollen, *Cistus ladaniferus*, osteoporosis

INTRODUCTION

Aging induces a decrease in bone mass. Osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem. The most dramatic expression of the disease is represented by fractures of the proximal femurs.[1–4] A decrease in bone mass may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional factors may prevent bone loss with increasing age.[5] Chemical compounds in food and plants that act on bone metabolism have not been fully clarified.

Recent studies have shown that isoflavones (including genistein and daidzen), which are contained in soybeans, have stimulatory effects on bone formation[6,7] and inhibitory effects on bone resorption,[8] thereby increasing bone mass. Menaquinone-7 (vitamin K_2), which is essential for the γ-carboxylation of osteocalcin of bone matrix protein, is abundant in fermented soybeans. Menaquinone-7 has been demonstrated to stimulate osteoblastic bone formation[9] and to inhibit osteoclastic bone resorption[10] *in vitro*. Carotenoids are present in fruit and vegetables. Of the various carotenoids (including β-cryptoxanthin, lutein, lycopene, and β-carotene), β-cryptoxanthin has been found to have a unique anabolic effect on bone calcification.[11] β-Cryptoxanthin has stimulatory effects on osteoblastic bone formation[12,13] and inhibitory effects on osteocalstic bone resorption[12,14] *in vitro*. The supplementation of isoflavones, menaquinone-7, and β-cryptoxanthin has preventive effects on bone loss induced by ovariectomy in rats, which is an animal model of os-

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teoporosis.[15–17] Food chemical factors thus play a role in bone health and may be important in the prevention of bone loss with aging.[18–20]

Bee pollen extract has been found to have anabolic effects on bone components in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* and *in vivo*.[21] Of various bee pollens, the water-solubilized extract obtained from *Cistus ladaniferus* (*C. ladaniferus*) has a marked effect on increasing bone calcification *in vitro*.[21] This study was undertaken to determine the effects of bee pollen extract obtained from *C. ladaniferus* on osteoclastic bone resorption *in vitro*.

MATERIALS AND METHODS

Chemicals —— Dulbecco's modified Eagle's medium (MEM) and penicillin-streptomycin solution (penicillin 5000 units/ml; penicillin 5000 μg/ml) were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (fraction V), synthetic human parathyroid hormone [PTH (1-34)], 1,25-dihydroxyvitamin D_3 (VD$_3$), prostaglandin E_2 (PGE$_2$), tumor necrosis factor-α (TNF-α), and lypopolysaccharide (LPS) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan).

Bee Pollen Extracts —— Bee pollen was obtained from *C. ladaniferus*. The powder of bee pollen (5 g) was suspended in distilled water (20 ml) and mixed vigorously, and the suspension was centrifuged at 10000 g in a refrigerated centrifuge for 20 min. The 10000 g supernatant fraction was collected and filtered, and the filtrate was freeze-dried. The powder of the water-solubilized extract was dissolved in ice-cold distilled water for use in experiments.

Animals —— Male Wistar rats (conventional) weighing 90–100 g (4 weeks old) or male mice (ddY strain; 6 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and 1.1% phosphorus and housed at room temperature of 25°C, with free access to distilled water.

Bone Culture —— The femurs were removed aseptically after bleeding, and were then soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal and -metaphyseal tissues were cut into small pieces. Diaphyseal or metaphyseal fragments were cultured in a 35 mm dish in 2.0 ml of medium consisting of Dulbecco's MEM (high glucose, 4.5 g/dl) supplemented with antibiotics (penicillin 100 units and streptomycin 100 μg/ml of medium).[22] In experiments, bone tissues were cultured in medium containing either vehicle or water-solubilized bee pollen extracts (10, 100, or 1000 μg/ml of medium) in the presence and absence of PTH (10^{-7} M), VD$_3$ (10^{-6} M), or PGE$_2$ (10^{-5} M). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air for 48 hr.

Analytical Procedures —— The diaphyseal and metaphyseal tissues were dried for 16 hr at 110°C. The calcium was determined using atomic absorption spectrophometry.[22] Calcium content in bone tissues was expressed as milligrams per gram of dry bone.

The concentration of glucose in the medium cultured with bone tissues for 48 hr was determined with the colorimetric method using *o*-toluidine.[23] The dry weight of the bone tissue was measured after extraction with 5.0% trichloroacetic acid, acetone, and diether ether. The medium glucose consumed by bone culture in 48 hr was expressed as milligrams of glucose per gram of dry bone tissue. The medium lactic acid was measured using the enzymatic method.[24] Data are expressed as milligrams of lactic acid per gram of dry bone tissue.

To assay bone tartrate-resistant acid phosphatase (TRACP), the diaphyseal or metaphyseal tissues were immersed in 3.0 ml of ice-cold 6.6 mM barbital buffer (pH 7.4), cut into small pieces, and disrupted for 60 sec with an ultrasonic device. The supernatant, centrifuged at $600 \times g$ for 5 min, was used to measure enzyme activity. TRACP activity was assayed using *p*-nitrophenylphosphate (pNPP) as substrate in an incubation medium (1.0 ml) containing 80 mM pNPP, 0.8 M Na-acetate (pH 5.8), 1.2 M KCl, 80 mM Na-tartrate, 8 mM ascorbic acid, 0.8 mM FeCl$_3$, and bone protein (5–10 μg/ml).[25] The *p*-nitrophenol liberated after 1 hr of incubation at 37°C was converted into *p*-nitrophenylate by the addition of 4.0 ml of 0.1 M NaOH, and the absorbance was immediately read at 405 nm. Enzyme activities were expressed as nanomoles of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentration was measured using the method of Lowry *et al*.[26]

Marrow Culture —— Bone marrow cells were isolated from mice, as reported elsewhere.[27] Briefly, bone ends of the femur were cut off, and the mar-

row cavity was flushed with 1 ml of α-MEM. The marrow cells were washed with α-MEM and cultured in the same medium containing 10% heat-inactivated fetal bovine serum at 1.0×10^7 cells/ml in 24-well plates (0.5 ml/well) in a water-saturated atomosphere containing 5% CO_2 and 95% air at 37°C. The cells were cultured for 3 days, then 0.2 ml of the old medium was replaced with fresh medium, the old medium was replaced with fresh medium and the cultures were maintained for an additional 4 days. The extract (10, 50, or 100 μg/ml) of bee pollen was added to the culture medium containing either vehicle, PTH (10^{-7} M), PGE$_2$ (10^{-5} M), TNF-α (10 ng/ml), or LPS (10 μg/ml), with an effective concentration at the beginning of the cultures and at the time with medium change.

Enzyme Histochemistry —— After being cultured for 7 days, cells adhering to the 24-well plates were stained for TRACP, a marker enzyme of osteoclasts.[25,28] Briefly, the cells were washed with Hank's balanced salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied according to the method of Burstone.[28] The fixed cells were incubated for 12 min at room temperature (25°C) in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma Chemical) as a substrate and red violet LB salt (Sigma Chemical) as a stain for the reaction product, in the presence of 10 mM sodium tartrate. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.

Statistical Analysis —— Data are expressed as the mean $\pm$ SEM. Statistical differences were analyzed using Student's t-test. p-Values < 0.05 were considered to indicate statistically significant differences. The analysis of variance (ANOVA) multiple comparison test was used to compare the treatment groups.

RESULTS

Effects of Cistus Bee Pollen Extract on Bone Resorption *in Vitro*

The effects of cistus bee pollen extract on the bone-resorbing factor-induced decrease in calcium content in the femoral-diaphyseal or -metaphyseal tissues of rats *in vitro* were examined. Bone tissues were cultured for 48 hr in medium containing either vehicle, bone-resorbing factor, or bone-resorbing factor plus cistus bee pollen extract (10, 100, or
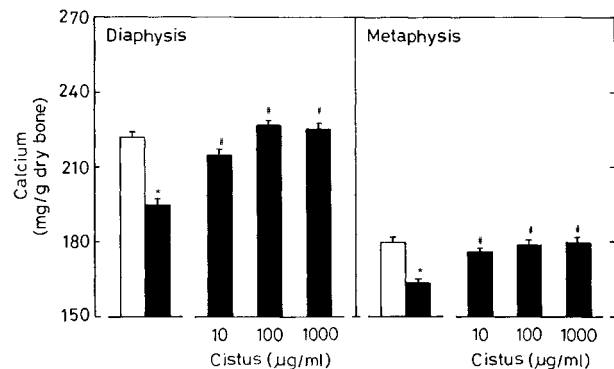

Fig. 1. Effects of Cistus Bee Pollen Extract on PTH-Induced Decrease in Calcium Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle, PTH (10^{-7} M), or PTH (10^{-7} M) plus bee pollen extracts (10, 100, or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean $\pm$ SEM of six rats. *$p < 0.01$ compared with the control (none) value. #$p < 0.01$ compared with the value obtained with PTH alone. White bar: control (none); black bars: PTH addition.
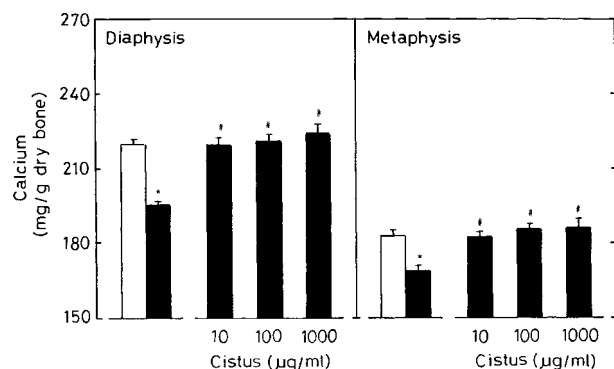

Fig. 2. Effects of Cistus Bee Pollen Extract on PGE$_2$-Induced Decrease in Calcium Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle, PGE$_2$ (10^{-5} M), or PGE$_2$ (10^{-5} M) plus bee pollen extracts (10, 100, or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean $\pm$ SEM of six rats. *$p < 0.01$ compared with the control (none) value. #$p < 0.01$ compared with the value obtained with PGE$_2$ alone. White bar: control (none); black bars: PGE$_2$ addition.

1000 μg/ml of medium). Calcium content in the diaphyseal or metaphyseal tissues was significantly decreased after culture with PTH (10^{-7} M) (Fig. 1), PGE$_2$ (10^{-5} M) (Fig. 2), or VD$_3$ (10^{-6} M) (Fig. 3). The effects of these bone-resorbing factors were completely inhibited in the presence of cistus bee pollen extract (10, 100, or 1000 μg/ml) (Figs. 1–3).

The effects of cistus bee pollen extract on the consumption of medium glucose and the production
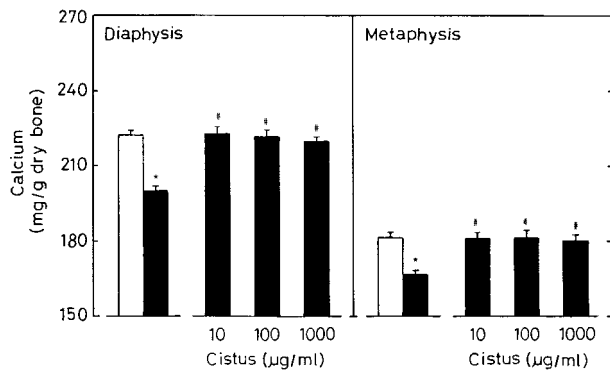
Fig. 3. Effects of Cistus Bee Pollen Extract on VD$_3$-Induced Decrease in Calcium Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle, VD$_3$ (10^{-6} M), or VD$_3$ (10^{-6} M) plus bee pollen extracts (10, 100, or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean ± SEM of six rats. *$p < 0.01$ compared with the control (none) value. #$p < 0.01$ compared with the value obtained with VD$_3$ alone. White bar: control (none); black bars: VD$_3$ addition.

Fig. 5. Effects of Cistus Bee Pollen Extract on PTH-Stimulated Lactic Acid Production in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle, PTH (10^{-7} M), or PTH (10^{-7} M) plus bee pollen extracts (10, 100, or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean ± SEM of six rats. *$p < 0.01$ compared with the control (none) value. #$p < 0.01$ compared with the value obtained with PTH alone. White bar: control (none); black bars: PTH addition.
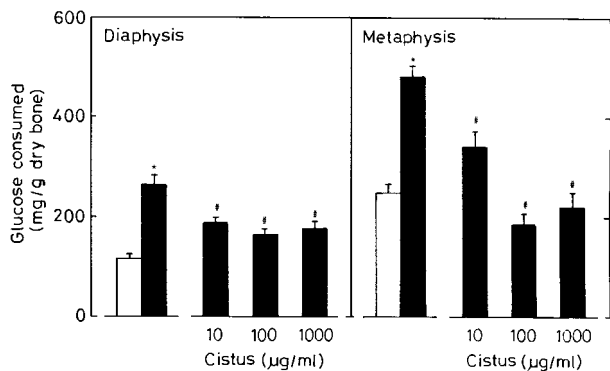

Fig. 4. Effects of Cistus Bee Pollen Extract on PTH-Stimulated Glucose Consumption in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle, PTH (10^{-7} M), or PTH (10^{-7} M) plus bee pollen extracts (10, 100, or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean ± SEM of six rats. *$p < 0.01$ compared with the control (none) value. #$p < 0.01$ compared with the value obtained with PTH alone. White bar: control (none); black bars: PTH addition.

Fig. 6. Effects of Cistus Bee Pollen Extract on PTH-Induced Increase in TRACP Activity in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle, PTH (10^{-7} M), or PTH (10^{-7} M) plus bee pollen extracts (10, 100, or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean ± SEM of six rats. *$p < 0.01$ compared with the control (none) value. #$p < 0.01$ compared with the value obtained with PTH alone. White bar, control (none); black bars, PTH addition.

of lactic acid in the femoral-diaphyseal or -metaphyseal tissues of rats *in vitro* was examined. Bone tissues were cultured for 48 hr in medium containing either vehicle, PTH (10^{-7} M) or PTH (10^{-7} M) plus cistus bee pollen extract (10, 100, or 1000 μg/ml). The presence of PTH (10^{-7} M) caused a significant increase in medium glucose consumption (Fig. 4) and lactic acid production (Fig. 5) in the diaphyseal or metaphyseal tissues. These increases were
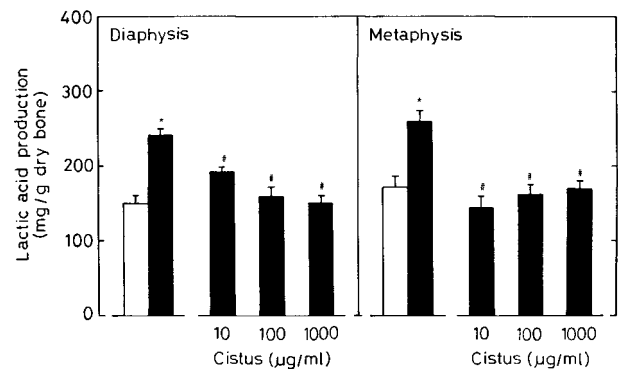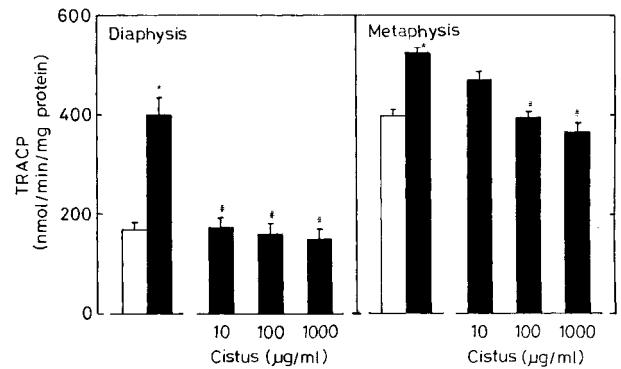
significantly inhibited with culture with cistus bee pollen extract (10, 100, or 1000 μg/ml) (Figs. 4 and 5).

The effects of cistus bee pollen extract on TRACP activity in the femoral-diaphyseal or -metaphyseal tissues of rats *in vitro* are shown in Fig. 6. The presence of PTH (10^{-7} M) caused a significantly increase in TRACP activity in the diaphyseal or metaphyseal tissues cultured for 48 hr. This increase
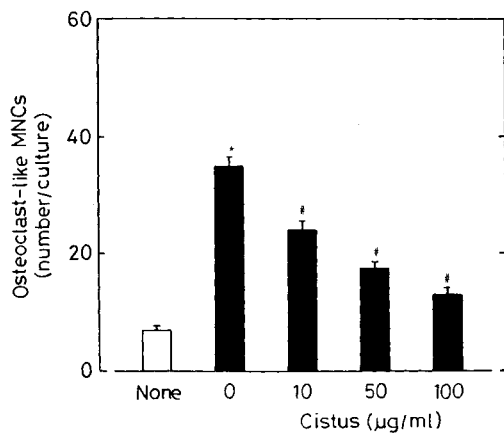
Fig. 7. Effects of Cistus Bee Pollen Extract on PTH-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

Mouse Marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-7} M), or PTH (10^{-7} M) plus bee pollen extracts (10, 50, or 100 μg/ml of medium) obtained from *C. ladanferus*. Each value is the mean $\pm$ SEM of six rats. $*p < 0.01$ compared with the control (none) value. $\#p < 0.01$ compared with the value obtained with PTH alone. White bar: control (none); black bars: PTH addition.
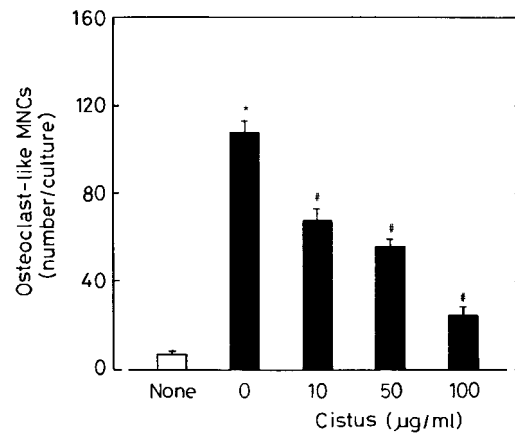

Fig. 8. Effects of Cistus Bee Pollen Extract on PGE_2-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

Mouse Marrow cells were cultured for 7 days in medium containing either vehicle, PGE_2 (10^{-5} M), or PGE_2 (10^{-5} M) plus bee pollen extracts (10, 50, or 100 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean $\pm$ SEM of six rats. $*p < 0.01$ compared with the control (none) value. $\#p < 0.01$ compared with the value obtained with PGE_2 alone. White bar: control (none); black bars: PGE_2 addition.

was significantly inhibited with culture of cistus bee pollen extract (100 or 1000 μg/ml).

Effects of Cistus Bee Pollen Extract on Osteoclast-Like Cell Formation *in Vitro*

The effects of cistus bee pollen extract on osteoclast-like cell formation were examined in mouse marrow culture *in vitro*. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, bone resorbing-factor, or bone-resorbing factor plus bee pollen extract (10, 50, or 100 μg/ml of medium). Culture with PTH (10^{-7} M) (Fig. 7), PGE_2 (10^{-5} M) (Fig. 8), TNF-α (10 ng/ml of medium) (Fig. 9), or LPS (10 μg/ml of medium) (Fig. 10) caused a significant increase in osteoclast-like cell formation. These increases were markedly inhibited in the presence of bee pollen extract (10, 50, or 100 μg/ml) (Figs. 7–10).

Fig. 9. Effects of Cistus Bee Pollen Extract on TNF-α-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

Mouse Marrow cells were cultured for 7 days in medium containing either vehicle, TNF-α (10 ng/ml of medium), or TNF-α (10 ng/ml) plus bee pollen extracts (10, 50, or 100 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean $\pm$ SEM of six rats. $*p < 0.01$ compared with the control (none) value. $\#p < 0.01$ compared with the value obtained with TNF-α alone. White bar, control (none); black bars, TNF-α addition.

DISCUSSION

Cistus Bee pollen extract has anabolic effects on bone components in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* and *in vivo*.[21] The active component in bee pollen is present in the water-solubilized extract.[21] This study demonstrated that the water-solubilized extract of bee pollen obtained from *C. ladaniferus* has inhibitory effects on
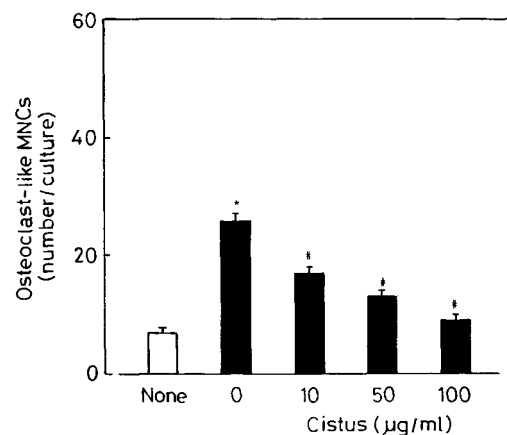
bone resorption in femoral tissues and osteoclast-like cell formation in bone marrow cell culture *in vitro*. Thus bee pollen extract has stimulatory effects on bone formation and inhibitory effects on bone resorption *in vitro*.

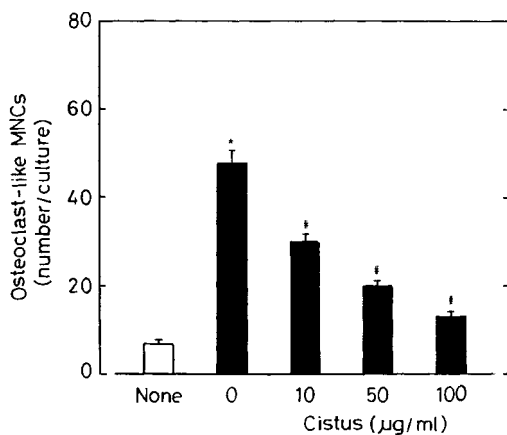Culture with the water-solubilized bee pollen

Fig. 10. Effects of Cistus Bee Pollen Extract on LPS-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

Mouse Marrow cells were cultured for 7 days in medium containing either vehicle, LPS (10 μg/ml of medium), or LPS (10 μg/ml of medium) plus bee pollen extracts (10, 50, or 100 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean ± SEM of six rats. *$p < 0.01$ compared with the control (none) value. #$p < 0.01$ compared with the value obtained with LPS alone. White bar: control (none); black bars: LPS addition.

extract had inhibitory effects on the decrease in calcium content in rat femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues induced in the presence of various bone-resorbing factors *in vitro*. This result suggests that bee pollen extract has inhibitory effects on bone resorption in rat femoral tissues *in vitro*. PTH is a bone-resoebing hormone and is important physiologically. PTH is known to stimulate bone resorption in organ culture.[29] PTH stimulates the excretion and synthesis of lysosomal enzymes and the extracellular release of acid by osteoclasts.[30] TRACP is a marker enzyme in osteoclastic cells.[31] Culture with bee pollen extract caused significant inhibitions of medium glucose consumption, lactic acid production, and TRACP activity in rat femoral-diaphyseal or -metaphyseal tissues *in vitro*. These results suggest that bee pollen extract has inhibitory effects on osteoclastic bone resorption *in vitro*.

Osteoclasts are formed from bone marrow cells.[32] Osteoclastogenesis is mediated through the action of the receptor activator of NF-λB ligand (RANKL) in the presence of macrophage colony-stimulating factor (M-CSF),[32,33] PTH increases the secretion of RANKL and M-CSF in osteoblasts and the hormone induces osteoclastogenesis.[32,33] Culture with bee pollen extract significantly inhibited the formation of osteoclast-like cells in mouse bone

marrow cells induced in the presence of PTH, PGE$_2$, TNF-α, or LPS, which stimulate osteoclastogenesis. This result demonstrates that bee pollen extract has inhibitory effects on osteoclastogenesis induced by various bone-resorbing factors *in vitro*. It is speculated that the active component in bee pollen extract has inhibitory effects on the secretion or action of RANKL.

Oral administration of bee pollen extract caused a significant increase in bone components in the femoral-diaphyseal and -metaphyseal tissues of rats *in vivo*.[21] The anabolic effects of bee pollen extract on bone components (including bone calcium) *in vivo* may result from its stimulatory effects on bone formation and its inhibitory effects on bone resorption. The supplementation of bee pollen extract may have preventive effects on bone loss with aging. The active components of bee pollen extract in stimulating bone formation and in inhibiting bone resorption remain to be elucidated.

In conclusion, it has been demonstrated that bee pollen extract obtained from *C. ladaniferus* has inhibitory effects on bone resorption in rat femoral-diaphyseal and -metaphyseal tissues *in vitro* and that the extract has inhibitory effects on osteoclastogenessis in mouse bone marrow cell culture *in vitro*.

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