Anabolic Effects of Bee Pollen *Cistus ladaniferus* Extract on Bone Components in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro* and *in Vivo*

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The effects of bee pollen extract on bone components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of rats *in vitro* and *in vivo* were investigated. Bone tissues were cultured for 48 hr in serum-free Dulbecco's modified Eagle's medium containing either vehicle or water- or ethanol-solubilized extracts (10, 100, or 1000 μg/ml of medium) obtained from the bee pollen of *Cistus ladaniferus*. Calcium content in the femoral-diaphyseal or -metaphyseal tissues was significantly increased in the presence of water-solubilized extract (100 or 1000 μg/ml) and ethanol-solubilized extract (1000 μg/ml). An increase was also observed in the presence of water-solubilized extract (100 μg/ml) obtained from *Fagopyrum esculentum*, *Camellia sinesis*, or *Brassica napus L*. Alkaline phosphatase activity and DNA content in the femoral-diaphyseal or -metaphyseal tissues *in vitro* were significantly increased in the presence of water-solubilized extract (100 or 1000 μg/ml) obtained from the bee pollen. The effects of the bee pollen extract (100 μg/ml) in increasing bone components were completely inhibited in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis, *in vitro*. Moreover, the calcium content and alkaline phosphatase activity in the femoral-diaphyseal or -metaphyseal tissues were significantly increased by the oral administration of water-solubilized extracts (5 or 10 mg/100 g body weight) obtained from the bee pollen of *Cistus ladaniferus* once daily for 7 days. The DNA content in the diaphyseal or metaphyseal tissues was significantly increased by the oral administration of water-solubilized extract (10 mg/100 g) of bee pollen cistus. The dose of 1.0 mg/100 g caused a significant increase in the diaphyseal and metaphyseal alkaline phosphatase activity or the metaphyseal DNA content *in vivo*. This study demonstrates that the extract of bee pollen has an anabolic effect on bone components in rats *in vitro* and *in vivo*.

Key words —— bone formation, bee pollen, *Cistus ladaniferus*, osteoporosis, rat femur

INTRODUCTION

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem.[1–3] A decrease in bone mass leads to bone fracture. Bone loss may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional supplements can prevent bone loss caused by increasing age.[4,5]

Food chemical factors may help to prevent bone loss with increasing age. Recent studies have shown that isoflavones (including genistein and daidzein), which are contained in soybeans,[5–8] menaquinone-7, an analogue of vitamin K_2 which is abundant in fermemted soybeans,[9–11] and β-cryptoxanthin, a carotenoid which is abundant in Satsuma mandarin (*Citrus unshiu* MARC.)[12–15] have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoblastic bone formation, thereby increasing bone mass. The intake of dietary factors has been shown to have a preventive effect on bone loss in animal models of osteoporosis.[16–18] Thus food factors may be useful in the prevention of osteoporosis.[19–21]

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This study was undertaken to determine the effects of bee pollen extract on bone components in the femoral tissues of rats, since the role of bee pollen in the prevention of bone loss with increasing age has not yet been clarified.

MATERIALS AND METHODS

Chemicals —— Dulbecco's modified Eagle's medium (MEM) (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (penicillin 5000 U/mg; streptomycin 5000 μg/ml) were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (fraction V) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan).

Bee Pollen and Propolis Extracts —— Bee pollen was obtained from *Cistus ladaniferus* (*C. ladaniferus*), *Brassica napus L.* (*B. napus L.*), *Fagopyrum esculentum* (*F. esculentum*), and *Camellia sinensis* (*C. sinensis*) L. O. Kuntze. 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.

The powder of bee pollen (20 g) was suspended in 99.5% ethanol (30 ml) and mixed vigorously, and the suspension was centrifuged at 800 *g* for 10 min. The 800 *g* supernatant fraction was collected and filtered, and the filtrate was freeze-dried. The powder of the ethanol-solubilized extract was dissolved in 99.5% ethanol for use in experiments.

Propolis was obtained from Brazil. The powder of propolis (20 g) was suspended in 99.5% ethanol (30 ml) and mixed vigorously, and the suspension was centrifuged at 800 *g* for 10 min. The 800 *g* supernatant fraction was collected and filtered. The filtrate was freeze-dried and dissolved in 99.5% ethanol for use in experiments. The precipitate from 800 *g* centrifugation after 99.5% ethanol extraction was suspended in distilled water and mixed vigorously. The suspension was centrifuged at 10000 *g* for 10 min, and the supernatant was collected and freeze-dried. The powder of the water-solubilized extract was dissolved in distilled water for use in experiments as a water-solubilized extract of propolis.

Animals —— Male Wistar rats (conventional) weighing 90–100 g (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed a 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 rats were killed by exsanguinations and the femurs were removed aseptically and soaked in ice-cold 0.25 M sucrose solution. The femurs were cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal or -metaphyseal tissues were cut into small pieces. Femoral-diaphyseal or -metaphyseal fragments were cultured for 24 hr in 35-mm dishes in 2.0 ml of medium consisting of Dulbecco's MEM (high glucose, 4.5g/dl) supplemented with 0.25% bovine serum albumin plus antibiotics (penicillin 100 units and streotomycin 100 μg/ml of medium).[22] In experiments, bone tissues were cultured for 48 hr in medium containing either vehicle or water- or ethanol-solubilized bee pollen extract.

In other experiments, culture medium was contained water- or ethanol-solubilized extract obtained from propolis. Cultures were maintained at 37°C in a water-saturated atomosphere containing 5% CO_2 and 95% air.

Administration Procedures —— The water-solubilized extract (1, 5, or 10 mg/ml 100 g body weight) obtained from the bee pollen of *C. ladaniferus* was orally administered to rats through a stomach tube once daily for 7 days. Control rats received distilled water (1.0 ml/100 g body weight) orally. The animals were killed 24 hr after the last administration by cardiac puncture under light ether anesthesia, and the blood and femur were removed immediately.

Analytial Procedures —— Blood samples were centrifuged for 30 min after collection, and the serum was separated and analyzed immediately. Serum calcium or inorganic phosphorus concentrations were determined using a kit (Wako Pure Chemical Industries).

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

To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in

3.0 ml of ice-cold barbital buffer 6.6 mM (pH 7.4), cut into small pieces, and disrupted for 60 sec with an ultrasonic device. The supernatant, centrifuged at 600 × *g* for 5 min, was used to measure enzyme activity. An enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.[23] Enzyme activity was expressed as micromoles of *p*-nitrophenol liberated per minute per milligram of protein. The protein concentration was determined using the method of Lowry *et al.*[24]

To measure the bone DNA content, the diaphyasel and metaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 hr after homogenization of the bone tissues.[25] After alkali extraction, the samples were centrifuged at 1000 × *g* for 5 min and the supernatant was collected. The DNA content was determined using the method of Ceriotti[26] and expressed as the amount of DNA (mg)/g of bone tissue wet weight.

Statistical Analysis —— The significance of differences between values was estimated using Student's *t*-test. A *p*-value of less 0.05 was considered to indicate a statistically significant difference.

RESULTS

Effects of Bee Pollen Extract on Bone Components *in Vitro*

The effects of bee pollen extract on calcium content in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* is shown in Fig. 1. When bone tissues were cultured for 48 hr in medium containing either vehicle or water- or ethanol-solubilized extracts (10, 100, or 1000 μg/ml of medium) obtained from the bee pollen of *C. ladaniferus*, the calcium content in the femoral-diaphyseal tissues was significantly increased in the presence of water- or ethanol-solubilized extracts. The metaphyseal calcium content was significantly increased in the presence of water-solubilized extract (100 or 1000 μg/ml) or ethanol-solubilized extract (1000 μg/ml). Thus bee pollen extract was found to have a stimulatory effect on calcium content in the femoral-diaphyseal and -metaphyseal tissues *in vitro*. The water-solubilized extract had more potent effects on bone calcium content as compared with those of the ethanol-solubilized extract.

The effects of various bee pollen extracts on calcium content in the femoral-diaphyseal tissues of rats *in vitro* are shown in Fig. 2. Diaphyseal calcium content was significantly increased in the presence of water-solubilized extracts obtained from the bee pollen of *F. esculentum*, *C. sinensis*, *B. napus L.*, or *C. ladaniferus*.

Femoral-diaphyseal tissues were cultured for 48 hr in medium containing either vehicle or water- or ethanol-solubilized extracts (10, 50, or 100 μg/ml of medium) obtained from propolis (Fig. 3). The presence of water-solubilized extract (100 μg/ml)

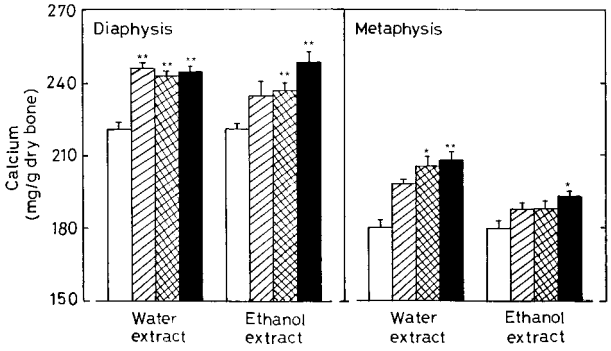

Fig. 1. Effects of Bee Pollen Extract on Calcium Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or water- or ethanol-solubilized extracts (10, 100, or 1000 μg/ml of medium) obtained from the bee pollen of *C. ladaniferus*. Each value is the mean ± S.E.M. of six rats. $*p < 0.05$ or $**p < 0.01$ compared with the control (none) value. White bars, control (none); hatched bars, 10 μg/ml; double hatched bars, 100 μg/ml; black bars, 1000 μg/ml.

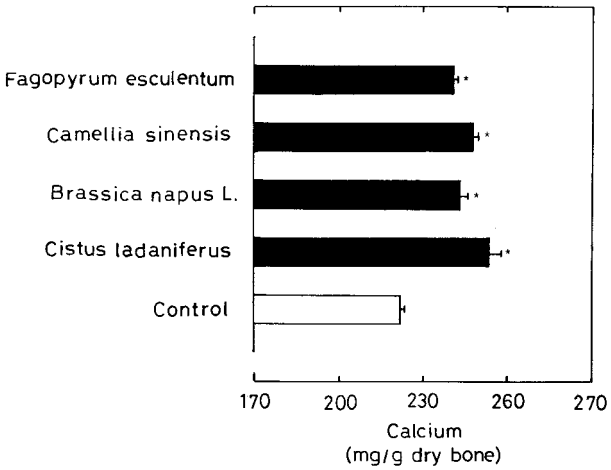

Fig. 2. Effects of Various Bee Pollen Extracts on Calcium Content in the Femoral-Diaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or water-solubilized extracts (100 μg/ml of medium) obtained from various bee pollens. Each value is the mean ± S.E.M. of six rats. $*p < 0.01$ compared with the control (none) value.

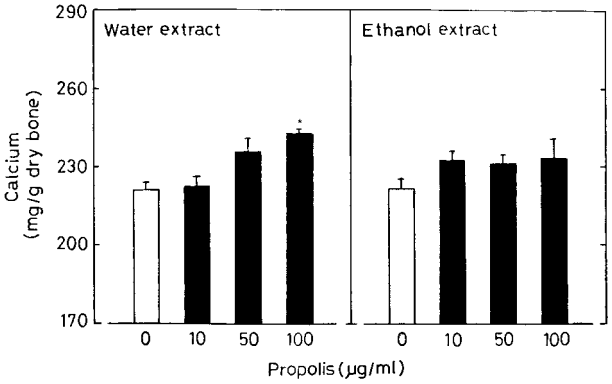

Fig. 3. Effects of Propolis Extract on Calcium Content in the Femoral-Diaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or water- or ethanol-solubilized extracts (10, 50, or 100 μg/ml of medium) obtained from propolis. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control (none) value.

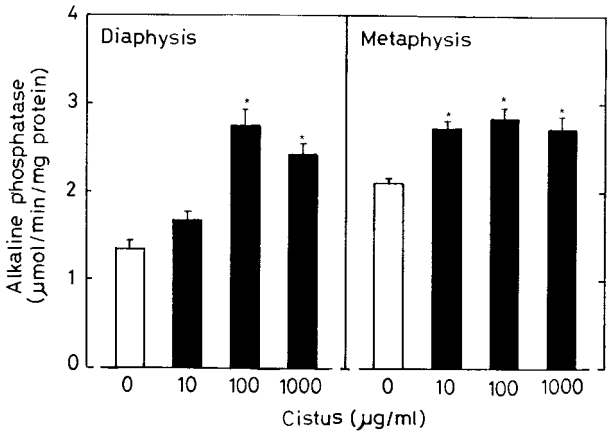

Fig. 4. Effects of Bee Pollen Extract on Alkaline Phosphatase Activity in the Femoral-Diaphyseal or -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or water-solubilized bee pollen extracts (10, 100, or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control (none) value.

caused a significant increase in the bone calcium content. Ethanol-solubilized extract (10, 50, or 100 μg/ml) did not have significant effects on the bone calcium content.

The effects of bee pollen extract on alkaline phosphatase activity and DNA content in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* are shown in Figs. 4 and 5 respectively. Alkaline phosphatase activity (Fig. 4) or DNA content (Fig. 5) in the femoral-diaphyseal or -metaphyseal

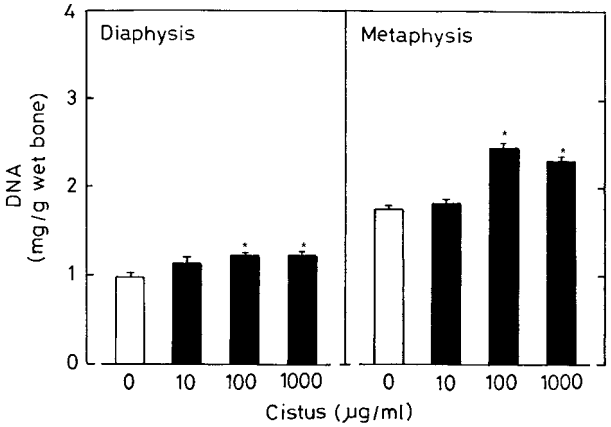

Fig. 5. Effects of Bee Pollen Extract on DNA Content in the Femoral-Diaphyseal or -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or water-solubilized bee pollen extracts (10, 100 or 1000 μg/ml of medium) obtained from *C. ladaniferus*. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control (none) value.

tissues was significantly increased in the presence of the water-solubilized extract (100 or 1000 μg/ml) obtained from the bee pollen of *C. ladaniferus*.

Femoral-diaphyseal or -metaphyseal tissues were cultured for 48 hr in the presence or absence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis (Fig. 6). The effects of the water-solubilized extract (100 μg/ml) obtained from bee pollen of *C. ladaniferus* in increasing the calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues were completely inhibited in the presence of cycloheximide (10^{-6} M).

Effects of Bee Pollen Extract on Bone Components in Rats *in Vivo*

Rats were orally administered water-solubilized extract (1, 5, or 10 mg/100 g body weight) obtained from the bee pollen of *C. ladaniferus* once daily for 7 days. The body weight of the animals or serum calcium and inorganic phosphorus concentrations were not significantly altered by the administration of the water-solubilized extract (data not shown).

The calcium content in the femoral-diaphyseal or -metaphyseal tissues was significantly increased by the administration of the water-solubilized extract (5 or 10 mg/100 g) obtained from the bee pollen of *C. ladaniferus* (Fig. 7). Alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues was significantly increased by the adminis-

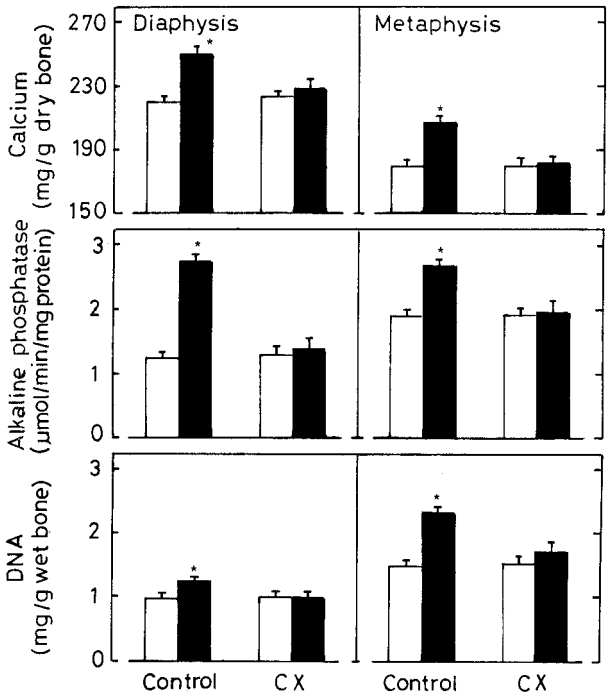

Fig. 6. Effects of Cycloheximide (CX) on the Bee Pollen Extract-Induced Increases in Components of the Femoral-Diaphyseal or -Metaphyseal Tissues of Rats *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or water-solubilized bee pollen extracts (100 μg/ml of medium) obtained from *C. ladaniferus* in the presence or absence of cycloheximide (10^{-6} M). Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control (none) value. White bars, control (none); black bars, cistus extract.

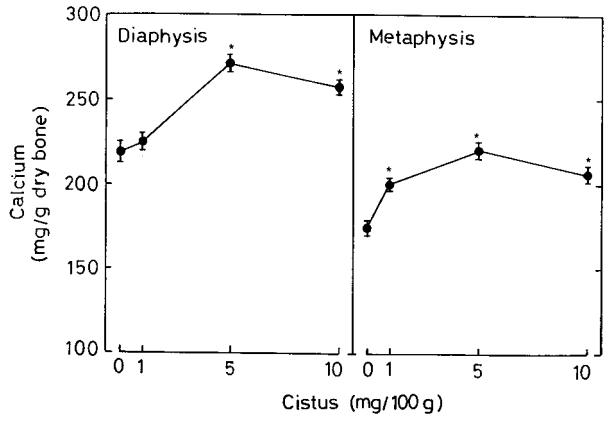

Fig. 7. Effects of Bee Pollen Extract on Calcium Content in the Femoral-Diaphyseal or -Metaphyseal Tissues of Rats *in Vivo*

Rats were orally administered either vehicle (distilled water) or water-solubilized bee pollen extracts (1, 5, or 10 mg/ml/100 g body weight) obtained from *C. ladaniferus* once daily for 7 days. Animals were killed 24 hr after the last administration. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control value.

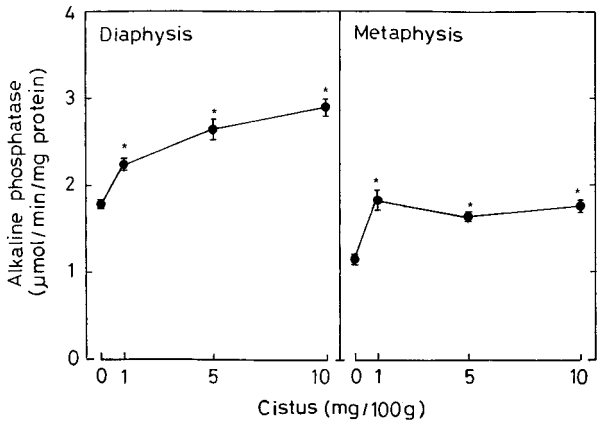

Fig. 8. Effects of Bee Pollen Extract on Alkaline Phosphatase Activity in the Femoral-Diaphyseal or -Metaphyseal Tissues of Rats *in Vivo*

Rats were orally administered either vehicle or water-solubilized bee pollen extracts (1, 5, or 10 mg/ml) obtained from *C. ladaniferus* once daily for 7 days. Animals were killed 24 hr after the last administration. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control value.

tration of the water-solubilized extract (1, 5, or 10 mg/100 g) obtained from the bee pollen (Fig. 8).

The DNA content in the femoral-diaphyseal tissues was significantly increased by the administration of the water-solubilized extract (10 mg/100 g) obtained from the bee pollen of *C. ladaniferus* (Fig. 9). Metaphyseal DNA content was also significantly increased by the administration of the water-solubilized extract (1, 5, or 10 mg/100 g).

DISCUSSION

Bee pollen extract was found to have anabolic effects on bone components in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* and *in vivo*. Of various bee pollens, the water-solubilized extract obtained from *C. ladaniferus* had a marked effect in increasing the bone calcium content. The ethanol-solubilized extract of bee pollen significantly increased the bone calcium content. However, the effects of the water-solubilized extract in increasing the bone calcium content were greater than those of the ethanol-solubilized extract. The active component in bee pollen was thus present in water-solubilized extract.

Alkaline phosphatase is an enzyme marker of osteoblasts and the enzyme participates in bone mineralization.[27] The DNA content in bone tissues is an index of the number of bone cells.[28] Culture with

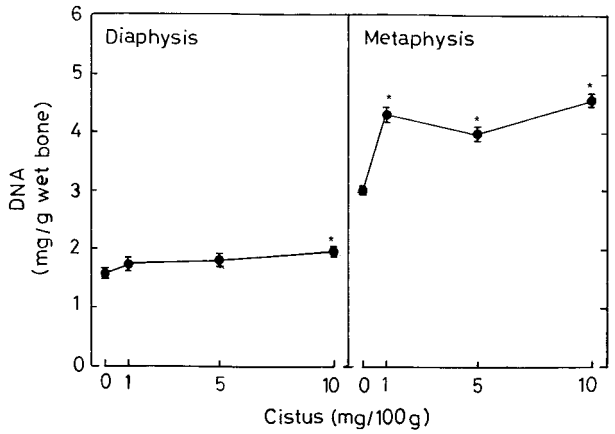

Fig. 9. Effects of Bee Pollen Extract on DNA Content in the Femoral-Diaphyseal or -Metaphyseal Tissues of Rats *in Vivo*

Rats were orally administered either vehicle (distilled water) or water-solubilized bee pollen extracts (1, 5, or 10 mg/ml) obtained from *C. ladaniferus* once daily for 7 days. Animals were killed 24 hr after the last administration. Each value is the mean ± S.E.M. of six rats. *$p < 0.01$ compared with the control value.

the extract obtained from the bee pollen of *C. ladaniferus* caused a significant increase in alkaline phosphatase activity and DNA content in the femoral-diaphyseal and -metaphyseal tissues *in vitro*. The increases were completely inhibited in the presence of cycloheximide, an inhibitor of protein synthesis. These observations suggest that the stimulatory effects of bee pollen extract on bone formation results from newly synthesized protein components *in vitro*.

The oral administration of the water-solubilized extract obtained from the bee pollen of *C. ladaniferus* to rats caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues, indicating that the extract exerts anabolic effects on bone components *in vivo*. The administration of bee pollen extract did not have a significant effect on the body weight of the animals or serum calcium and inorganic phosphorus concentrations, which are regulated by calcium-regulating hormones. It is speculated that the active component in the water-solubilized extract obtained from the bee pollen of *C. ladaniferus* is transported in the intestine, and acts on osteoblastic cells in bone tissues. If the active component is transported in the intestine, it may not be a high molecular-weight compound. The active component in bee pollen extract remains to be identified.

Bee pollen is used as a nutrient factor. The supplemental intake of dietary bee pollen may help to prevent bone loss with increasing age. This should be determined using animal models of osteoporosis *in vivo*.

In conclusion, it has been demonstrated that the water-solubilized extract obtained from the bee pollen of *C. ladaniferus* has anabolic effects on bone components in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* and *in vivo*.

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