Preventive Effects of Bee Pollen *Cistus ladaniferus* Extract on Bone Loss in Streptozotocin-Diabetic Rats *In Vivo*

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The effect of bee pollen cistus extract on serum and bone biochemical components in streptozotocin (STZ)-diabetic rats was investigated. The water-solubilized extracts were obtained from the bee pollen of *Cistus ladaniferus*. Rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and then the animals were orally administered water-solubilized extract (5, 10, or 20 mg/100 g body weight) of bee pollen cistus once daily for 14 days. The administration of STZ caused a significant decrease in body weight and a significant increase in serum glucose, triglyceride, and calcium levels, indicating a diabetic state. These alterations were significantly prevented by the administration of the extract (5, 10, or 20 mg/100 g). Serum inorganic phosphorus concentration was significantly decreased in STZ-diabetic rats, and the decrease was significantly prevented after administration of the extract of 10 or 20 mg/100 g. Calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues were significantly decreased in STZ-diabetic rats. These decrease were significantly prevented after administration of the extract of 10 or 20 mg/100 g. The disphyseal DNA content was also significantly decreased in STZ-diabetic rats. This decrease was significantly prevented after the administration of the extract of 10 or 20 mg/100 g. This study demonstrates that the intake of bee pollen cistus extract has preventive effects on bone loss in STZ-diabetic rats, and that the intake has partial restorative effects on serum biochemical findings with the diabetic state.

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

INTRODUCTION

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem.[1–4] 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 may prevent bone loss with increasing age.[5–7] Micronutrients and phytochemicals are found in vegetables and fruits. Our recent studies have shown that isoflavones,[8–10] vitamin K_2,[11–13] and carotenoid β-cryptoxanthin[14–17] have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption, thereby increasing bone mass. Thus food chemical factors may have a useful role in the prevention of osteoporosis with aging.

We demonstrated that the water-solubilized extract of bee pollen *Cistus ladaniferus* has anabolic effects on bone components in rats *in vitro* and *in vivo*.[18] The extract of bee pollen cistus has stimulatory effects on bone formation and inhibitory effects on bone resorption *in vitro*.[18,19] The extract stimulates bone calcification as potently as propolis.[18] Royal jelly does not have an inhibitory effect on osteoclastic cell formation,[20] so that the effect of bee pollen on bone metabolism may thus be unique among bee-related products. The active component of bee pollen cistus extract, which stimulates bone formation and inhibits osteoclastic bone resorptopn, has been shown to be a fraction with molecular weight of less than 1000.[21]

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This study was undertaken to determine whether the intake of bee pollen cistus extract has preventive effects on bone loss induced in the diabetic state.[22,23]

MATERIALS AND METHODS

Chemicals —— Streptozotocin (STZ) was 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 Extracts —— Bee pollen was obtained from *Cistus ladaniferus*.[18] The power of bee pollen cistus (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 105–115 g (5 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.

Administration Procedures —— STZ was dissolved in 50 mM sodium citrate (pH 4.5) solution containing 150 mM NaCl.[22] The solution (6.0 mg/0.5 ml/100 g body weight) was subcutaneously administered to rats, and 14 days later the animals were killed by exsanguination. The water-solubilized extract (5, 10, or 20 mg/ml/100 g body weight) obtained from the bee pollen of *Cistus ladaniferus* was orally administered to rats through a stomach tube once daily for 14 days. The extract was orally administered 3 hr after the administration of STZ (6.0 mg/100 g). Rats were killed 24 hr after the last administration of bee pollen sistus extract, and the blood and femur were removed immediately.

Analytical Procedures —— Blood samples obtained by cardiac puncture were centrifuged 30 min after collection, and the serum was separated. Serum was frozen at −80°C until assay. Serum glucose, triglyceride, calcium, and inorganic phopsphorus concentrations were determined using an assay kit (Wako Pure Chemical Industries).

The diaphyseal or metaphyseal tissues were dried for 16 hr at 110°C. Calcium was determined using atomic absorption spectrophotometry.[24] Calcium content in bone tissues was expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the diaphyseal or metaphyseal tissues were immersed in 3.0 ml 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 $600 \times g$ for 5 min was used to measure enzyme activity. Enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined using the method of Walter and Schutt.[25] Enzyme activity was expressed as micromole of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentration was determined using the method of Lowry *et al.*[26]

To measure bone DNA content, the diaphyseal or metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr after homogenization of the bone tissues.[27] After alkaline extraction, the samples were centrifuged at $1000 \times g$ for 5 min, and the supernatant was determined using the method of Ceriotti[28] and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

Statistical Analysis —— Data are expressed as the mean ± standard error of the mean (S.E.M.). Statistical differences were analyzed using Student's *t*-test. p-value < 0.05 was considered to indicate a statistically significant difference. The analysis of variance (ANOVA) multiple comparison test was used to compare the treatment groups.

RESULTS

Effects of Administration of Bee Pollen Cistus Extract on Serum Biochemical Components in STZ-diabetic Rats

Rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and the animals were orally administered water-solubilized extract (5, 10, or 20 mg/100 g body weight) obtained from bee pollen of *Cistus ladaniferus* once daily for 14 days. The body weight of animals was significantly decreased at 14 days after administration of STZ, but this reduction was significantly prevented after administration of the extract (5, 10, or 20 mg/100 g) for 14 days (Fig. 1).

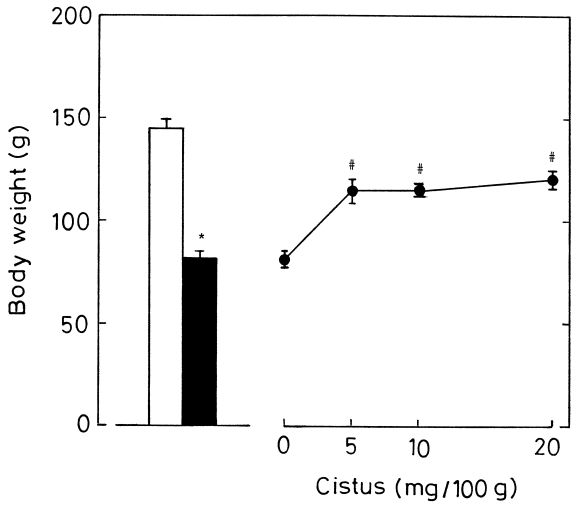

Fig. 1. Effects of Administration of Bee Pollen Cistus Extract on the Change in Body Weight in STZ-diabetic Rats

Rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and 3 hr later the animals were orally administered either vehicle (distilled water) or water-solubilized bee pollen extracts (5, 10, or 20 mg/ml/100 g body weight) obtained from *Cistus ladaniferus* once daily for 14 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 (none) value. $^{\#}p < 0.01$ compared with the control value from STZ treatment. White bar, control, black bar, bee pollen cistus extract.

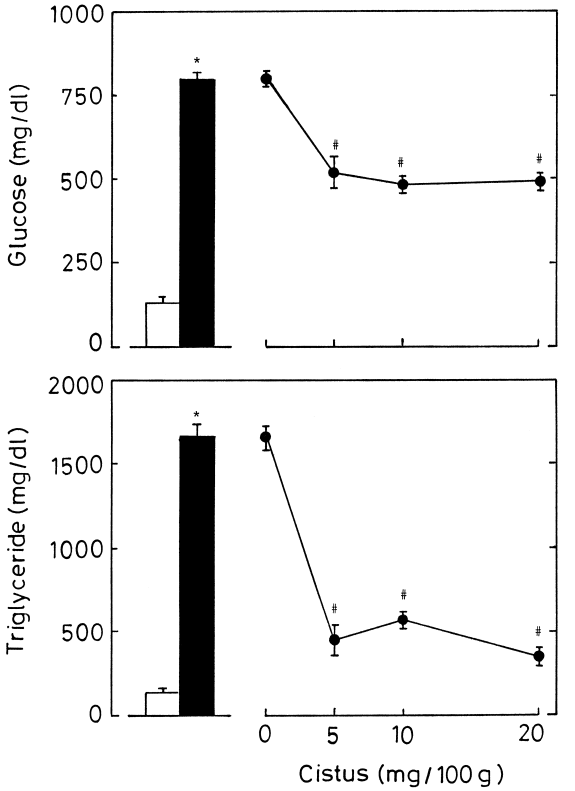

Fig. 2. Effects of Administration of Bee Pollen Cistus Extract on the Change in Serum Glucose and Triglyceride Concentrations in STZ-diabetic Rats

The procedure of administration is described in the legend of Fig. 1. Each value is the mean ± S.E.M. of six rats. $^{*}p < 0.01$ compared with the control (none) value. $^{\#}p < 0.01$ compared with the control value from STZ treatment. White bars, control, black bars, bee pollen cistus extract.

The serum glucose and triglyceride levels were markedly elevated in STZ-administered rats, indicating that the administration induces a diabetic state. These increases were significantly prevented after the administration of bee pollen cistus extract (5, 10, or 20 mg/100 g) for 14 days (Fig. 2).

The serum calcium levels were significantly increased after STZ administration (Fig. 3), and the increase was significantly prevented after administration of the extract (5, 10, or 20 mg/100 g) for 14 days. Serum inorganic phosphorus levels were also significantly decreased 14 days after STZ administration (Fig. 3), and this decrease was significantly prevented 14 days after administration of the extract (10 or 20 mg/100 g) for 14 days.

Effects of Administration of Bee Pollen Cistus Extract on Bone Components in STZ-diabetic Rats

The effects of administration of bee pollen cistus extract on bone components in the femoral-diaphyseal and -metaphyseal tissues of STZ-diabetic rats were examined. Calcium content in these tissues was significantly decreased 14 days after the administration of STZ (6.0 mg/100 g) (Fig. 4), and the decrease was significantly prevented after administration of the extract (5, 10, or 20 mg/100 g) for 14 days.

Alkaline phosphatase activity in these tissues was also significantly decreased in STZ-diabetic rats (Fig. 5), and the decrease was significantly prevented after administration of the extract (10 or 20 mg/100 g) for 14 days. The dose of extract (5 mg/100 g) for 14 days had a significant preventive effect on the decrease in diaphyseal alkaline phosphatase activity in STZ-diabetic rats.

DNA content in the femoral-diaphyseal tissues was significantly decreased in STZ-diabetic rats (Fig. 6), and the decrease was significantly prevented after administration of bee pollen cistus extract (10 or 20 g/100 g) for 14 days.

DISCUSSION

The water-solubilized extract obtained from the bee pollen of *Cistus ladaniferus* has been shown to

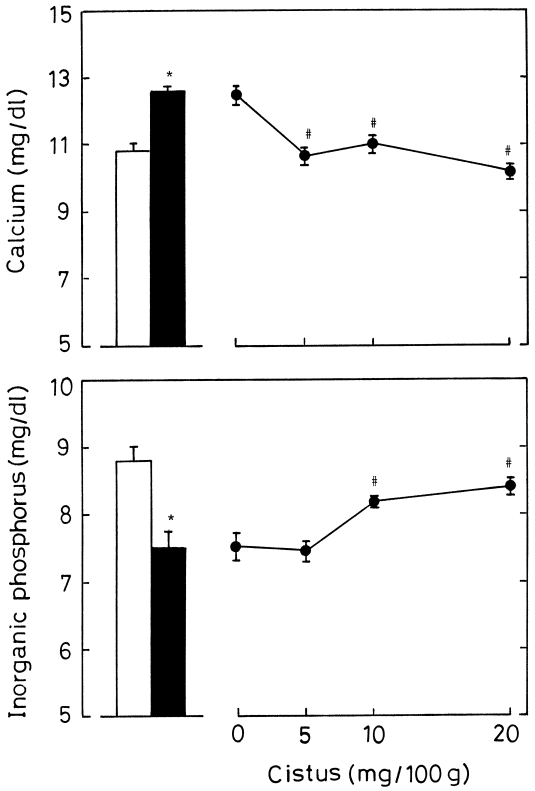

Fig. 3. Effect of Administration of Bee Pollen Cistus Extract on the Change in Serum Calcium and Inorganic Phosphorus Concentrations in STZ-diabetic Rats

The procedure of administration is described in the legend of Fig. 1. Each value is the mean ± S.E.M. of six rats. $^{*}p < 0.01$ compared with the control (none) value. $^{\#}p < 0.01$ compared with the control value from STZ treatment. White bars, control, black bars, bee pollen cistus extract.

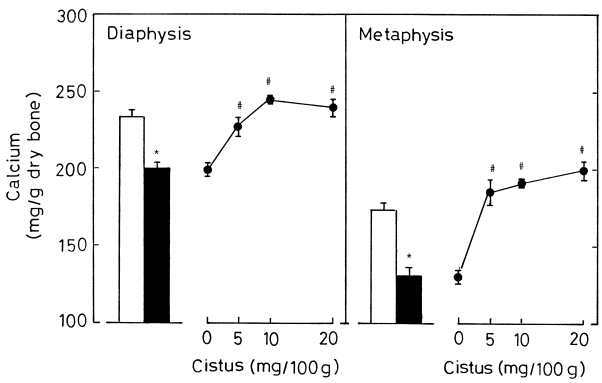

Fig. 4. Effects of Administration of Bee Pollen Cistus Extract on the Change in Calcium Content in the Femoral-diaphyseal and -metaphyseal Tissues of Rats

The procedure of administration is described in the legend of Fig. 1. Each value is the mean ± S.E.M. of six rats. $^{*}p < 0.01$ compared with the control (none) value. $^{\#}p < 0.01$ compared with the control value from STZ treatment. White bars, control, black bars, bee pollen cistus extract.

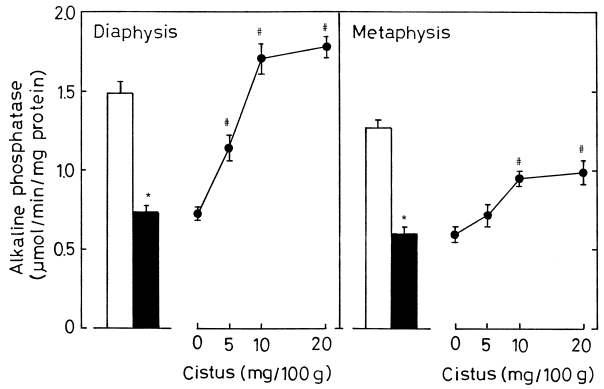

Fig. 5. Effects of Administration of Bee Pollen Cistus Extract on the Change in Alkaline Phosphatase Activity in the Femoral-diaphyseal and -metaphyseal Tissues of Rats

The procedure of administration is described in the legend of Fig. 1. Each value is the mean ± S.E.M. of six rats. $^{*}p < 0.01$ compared with the control (none) value. $^{\#}p < 0.01$ compared with the control value from STZ treatment. White bars, control, black bars, bee pollen cistus extract.

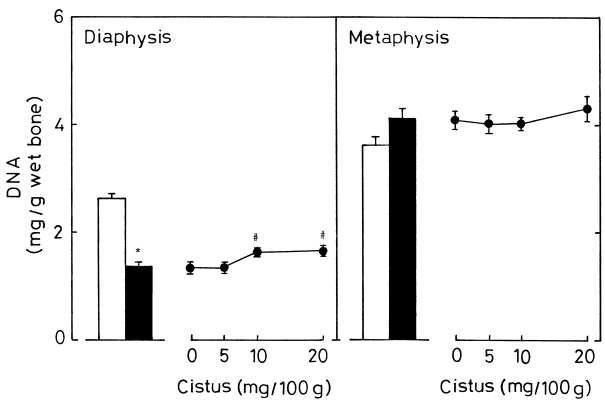

Fig. 6. Effect of Administration of Bee Pollen Cistus Extract on the Change in DNA Content in the Femoral-diaphyseal and -metaphyseal Tissues of Rats

The procedure of administration is described in the legend of Fig. 1. Each value is the mean ± S.E.M. of six rats. $^{*}p < 0.01$ compared with the control (none) value. $^{\#}p < 0.01$ compared with the control value from STZ treatment. White bars, control, black bars, bee pollen cistus extract.

have a stimulatory effect on bone formation and an inhibitory effect on bone resorption *in vitro*,[18, 19] thereby increasing bone mass. Diabetes has been shown to induce bone loss.[22, 23, 29] The oral administration of bee pollen cistus extract to STZ-diabetic rats was found to have preventive effects on bone loss with diabetes *in vivo*, suggesting that dietary intake of the extract has a preventive effect on bone loss in the pathophysiologic state.

The serum calcium level was found to increase in STZ-diabetic rats. Intestinal calcium absorption has been shown to be impaired in the diabetic

state.[30,31] The increase in serum calcium concentration in STZ-diabetic rats may result from the release of calcium from bone tissues; the femoral calcium content was found to decrease markedly in STZ-diabetic rats. The oral administration of the extract to these rats had a significant preventive effect on hypercalcemia and bone calcium loss in the diabetic state; intake of the extract may thus have an inhibitory effect on bone resorption in these rats.

Alkaline phosphatase activity in the femoral tissues was found to decrease in STZ-diabetic rats. The enzyme participates in osteoblastic mineralization.[32] The femoral alkaline phosphatase activity was significantly decreased in these rats, suggesting that osteoblastic bone mineralization is impaired in the diabetic state. The decrease in femoral alkaline phosphatase activity in STZ-diabetic rats was significantly prevented after the administration of bee pollen cistus extract. Presumably, intake of the extract has a stimulatory effect on osteoblastic bone formation in this state.

An active component with a molecular weight of less than 1000 is present in bee pollen cistus extract.[21] This active component has been shown to have a stimulatory effect on bone formation and an inhibitory effect on bone resorption *in vitro*.[18,19] These effects of bee pollen cistus extract are also seen in the STZ-diabetic state.

The oral administration of bee pollen cistus extract to STZ-diabetic rats was found to have a preventive effect on the decrease in body weight and the increase in serum glucose and triglyceride levels induced in the diabetic state. This is a novel finding. Intake of this extract has a partial restorative effect on serum biochemical findings involving diabetes *in vivo*. Whether the active component of the extract in preventing bone loss induced by diabetes is identical to the component which can prevent the elevation of serum glucose and triglyceride levels with diabetes remains to be elucidated.

In conclusion, it has been shown that the intake of bee pollen cistus extract by diabetic rats has a preventive effect on diabetic-induced bone loss, and also has a partial preventive effect on the increase in serum glucose and triglyceride levels with diabetes *in vivo.*

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