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Effect of Pine Pollen Extract on Experimental Chronic Arthritis

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The effects of pine pollen extract (PE) on Freund's complete adjuvant (FCA)-induced arthritis and collagen-induced arthritis (CIA) were investigated. The oral administration of PE (100 and 200 mg/kg per day) for 21 days after subcutaneous immunization with FCA, significantly reduced hindpaw swelling and the production of inflammatory cytokines (TNF-α, IL-1β and IL-6) compared with the FCA-induced arthritis group. Treatment with the PE (100 mg/kg) also decreased the serum levels of LDL-cholesterol and increased HDL-cholesterol contents compared with those of the arthritis group. Since CIA is a model of some types of human autoimmune rheumatoid arthritis (RA), the study examined whether PE is efficacious against CIA in mice and investigated the possible antioxidant potential of PE on CIA. Arthritis in DBA/1J mice was induced by subcutaneous immunization with bovine type II collagen. PE (100 and 200 mg/kg) was orally administered once daily for 49 days after initial immunization with type II collagen. The arthritis score and paw edema were markedly suppressed in the groups treated with PE. Moreover, administration of PE (100 mg/kg) for 49 days reduced the serum levels of rheumatoid factor, anti-type II collagen antibody, TNF-α, IL-1β, IL-6, protein carbonyl, advanced glycation endproducts, malondialdehyde and LDL-cholesterol compared with that of CIA mice. These results suggest that the pine pollen might be beneficial in the treatment of chronic inflammatory disorders. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: pine pollen; chronic inflammation; FCA-induced arthritis; collagen-induced arthritis.

INTRODUCTION

Inflammatory diseases are very common throughout the world. Inflammation in the joints leads to pannus formation, an array of infiltrated lymphocytes and fibrin in the joints. With time, painful erosion of the cartilage in the joint takes place. In the synovitis of rheumatoid arthritis (RA), leukocytes that have migrated into synovial tissue generate cytokines (e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6), which enhance inflammation (Feldmann *et al.*, 1996). These cytokines activate endothelial cells and circulating leukocytes and cause expression or upregulation of several adhesion molecules (Collins *et al.*, 1995), which are known to play important roles in the adhesion of leukocytes to vascular endothelium and their trans-endothelial migration into inflammatory sites. Sub- or intradermal injection of Freund's complete adjuvant (FCA) has been used widely to induce a RA-like inflammation in animals. Type II collagen induced arthritis (CIA) in DBA/1J mice is a known model of autoimmune disease, in which the joint pathology associated with CIA is similar to that observed in RA patients. CIA is associated with the activation of cell-mediated immunity, which results in the secretion of inflammatory cytokines such as IL-1β, TNF-α and IL-6 that are known to contribute to articular degradation (Koch *et al.*, 1995).

Although rheumatism is one of the oldest known diseases of mankind, affecting the majority of the population, no substantial progress has been made in achieving a permanent cure. The greatest disadvantage in the presently available potent synthetic drugs lies in their toxicity and the reappearance of symptoms after discontinuation. Antiinflammatory drugs transiently reduce symptoms, yet the disease progresses over time. In addition, non-steroidal antiinflammatory drugs induce gastric or hepatic toxicity (Singh, 1998). Other disease-modifying drugs, such as methotrexate (Bannwarth *et al.*, 1994), suffer from toxicities associated with antineoplastic drugs, which limits their long-term usefulness. Therefore, over the past few years many research studies have focused on edible plants with therapeutic properties. As a kind of Chinese traditional medicine, pine pollen, which is the male spore of pine tree has been used as a drug and food for thousands of years. Different from bee pollen, pine pollen is collected artificially, and it has the characteristics of a single pollen source, pure quality and stable component. Pine pollen powder is rich in many kinds of body-demanding amino acids, minerals, vitamins, enzymes and flavonoids (Wang *et al.*, 2005). Pollen lipids of a pine species were shown to have a marked inhibition of platelet activating factor activity (Siafaka-Kapadai *et al.*, 1986). It was found that vitamin D (D2, D3) was present in the pine pollen (Saden-Krehula and Tajic, 1987). Testosterone, epitestosterone and androstenedione were found in the pollen of Scotch pine *P. silvestris* L. (Saden-Krehula *et al.*, 1971).

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The objective of this study was to evaluate the anti-inflammatory potential of pine pollen in the FCA-induced arthritis and CIA models. In the FCA-induced arthritis model, the effects of a 70% ethanol extract of pine pollen (PE), orally administered daily for 21 days, on the inflammatory reactions were investigated. To further investigate the antirheumatic effect of PE, the effect of PE administration daily for 49 days on established CIA in DBA/1J mice was examined. The levels of lipid profiles and oxidation products (PCO, AGE and MDA) were measured in the serum of CIA mice treated with PE.

MATERIALS AND METHODS

Preparation of extract. The pine pollen collected at Kangwondo, Korea, was extracted three times with 70% ethanol at room temperatures for 3 days each and the combined extracts were concentrated *in vacuo* and then freeze dried (yield 8% w/w).

Freund complete adjuvant (FCA)-induced arthritis. Male ICR mice (6 weeks old) were purchased from Jungang Lab Animal Inc (Korea). These animals were maintained under constant temperature (24 °C), with a 12 h light–dark cycle, relative humidity 40–70% and allowed food and water *ad libitum*. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD).

Induction of arthritis by FCA. Mice were injected with 0.1 mL of FCA (1 mg/mL in mineral oil; Sigma, St Louis, MO) in the right posterior plantar region (Corsi *et al.*, 1999). Mice were randomly divided into four groups ($n = 7$), these being a normal untreated group (Group I), a control group injected with FCA and an orally administered vehicle (Group II), and treated groups (Group III and IV) injected with FCA and orally administered pine pollen extract (100 and 200 mg/kg) daily for 21 days. The pad thicknesses of the hind paws were measured with a dial thickness gauge (Mitutoyo, Japan) before and on day 21 after the FCA injection, and the difference in the thicknesses was calculated. The degree of foot-pad swelling was expressed as an increase in foot-pad thickness (mm). On day 22 after adjuvant injection, the body weights of the mice fasted overnight was obtained on groups of mice before killing. The liver, spleen and kidney were removed and weighed. Blood was collected by heart puncture and the serum was collected by centrifuging at $2000 \times \mathbf{g}$ for 15 min and stored at −80 °C until required. Protein was measured by the Bio-Rad Bradford protein assay kit.

Type II collagen-induced arthritis. Female DBA/1J mice, aged 6 weeks, were purchased from Japan SLC Inc. (Shizuoka, Japan). Animals received food and water *ad libitum* and were acclimatized to standard laboratory conditions (25 ± 3 °C, 55% humidity and 12 h light–dark cycle) for 7 days.

Induction of arthritis by Type II collagen. Type II collagen (Sigma, St Louis, MO, USA) was dissolved overnight at 4 °C in 50 mM acetic acid to 2 mg/mL. This solution was then emulsified in an equal volume of FCA in an ice-cold water bath. DBA/1J mice were immunized by the intradermal injection of 0.1 mL of this emulsion into the right hindpaw. Mice were boosted using the same schedule 21 days later. Pine pollen extract (100 and 200 mg/kg) was orally administered once daily for 49 days after initial immunization with type II collagen. The clinical severity was characterized by palpation and observations of joint properties and inflammation of surrounding tissue and it was assessed on a scale of 0–3, using a previously published scoring system (Williams *et al.*, 1992): 0, normal; 1, slight swelling and/or erythema of the fingers; 2, pronounced edematous swelling 3, joint rigidity with edematous swelling or joint ankylosis. Scores of 1 and 2 mainly reflect reversible edematous inflammation, but a score of 3 reflects irreversible components such as established joint ankylosis. Edema in the hind paw was measured before the initial injection of type II collagen and after the booster injection, using a dial thickness gauge. Body weight, clinical score and hindpaw swelling were monitored weekly over a period of 49 days. Blood was collected by heart puncture on day 50. The serum was collected by centrifuging at $2000 \times \mathbf{g}$ for 15 min and stored at −80 °C until required. Protein was measured by the Bio-Rad Bradford protein assay kit.

Biochemical analysis in serum. Rheumatoid factor (RF) level was assayed using mouse RF enzyme-linked immunosorbent assay (ELISA) kit (Alpha Diagnostic, USA). Anti-type II collagen IgG was measured by the ELISA kit (Chondrex, USA). Cytokine levels were determined using ELISA kits for murine TNF-α, IL-1β and IL-6 (R&D Systems, USA). The concentrations of triglyceride (TG), total cholesterol and high density lipoprotein (HDL)-cholesterol in serum were determined enzymatically using commercial available kit reagents (Boehringer, Mannheim, Germany). Low density lipoprotein (LDL)-cholesterol was calculated by Friedewald formula: LDL cholesterol = total cholesterol – HDL cholesterol – TG/5 (Tietz, 1986). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and creatinine level were assayed using a commercial kit (YD Diagnostics. Korea). The protein carbonyl assay kit (Cayman Chemical Co. MI, USA) was used to measure the protein carbonyl (PCO) content. This kit utilizes the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. Advanced glycation endproducts (AGE) levels were determined spectrofluorometrically as described by Kalousava *et al.* (2002). Samples were diluted 1:50 with PBS (pH 7.4) and the fluorescence intensities were recorded at 440 nm using excitation at 350 nm. Fluorescence intensities are expressed in arbitrary units as AU/mg protein. Lipid peroxidation (as MDA) levels were measured with the thiobarbituric acid reaction by the method of Placer *et al.* (1966) as described in previous studies.

Statistical analysis. The results are expressed as the mean ± SEM ($n = 5$). Statistical significance was determined by an analysis of variance and subsequent Dunnett's test ($p < 0.05$). The analysis was performed using the SAS statistical software.

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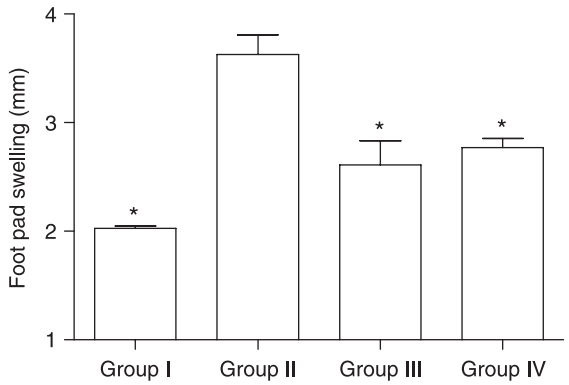

Figure 1. Effects of pine pollen extract on the FCA-induced paw edema in mouse after 21 days. Group I, non-immunized normal mice; Group II, arthritic mice not treated with sample; Group III, arthritic animals treated with pine pollen extract (100 mg/kg); Group IV, arthritic animals treated with pine pollen extract (200 mg/kg). Results are expressed as mean ± SEM (n = 7). * Significantly different from Group II, $p < 0.05$.

RESULTS AND DISCUSSION

Effects of pine pollen extract on the FCA-induced arthritis

The model of adjuvant-induced arthritis in the mouse has been used for many years for the evaluation of antiarthritic/antiinflammatory agent and is well characterized (Silvan *et al.*, 1997). FCA injection results in a localized monoarthritis enabling the study of the arthritic lesion without complicating factors of poor animal mobility, altered weight gain and systemic disease. The syndrome is characterized by the development of chronic joint inflammation, with cell proliferation, synovium enlargement, pannus formation and destruction of joint cartilage (Hang *et al.*, 1982). In the present study, arthritis was induced by a FCA injection. PE was administered orally at doses of either 100 or 200 mg/kg daily for 21 days. The results in Fig. 1 show that FCA injection resulted in a significant increase in paw thickness after 21 days and PE caused significant ($p < 0.05$) inhibition of paw edema compared with the FCA-induced arthritis group. The concentration and/or activity of many cytokines increase dramatically in response to stressful or pathophysiological conditions (Elenkov and Chrousos, 1999). The excessive production of proinflammatory cytokines, including TNF-α, IL-1β and IL-6, has been implicated as a major factor in the pathogenesis of diseases. FCA treatment induced pronounced changes in the serum levels of pro-inflammatory cytokines after 21 days compared with normal mice. The TNF-α, IL-1β and IL-6 levels in the PE groups were significantly ($p < 0.05$) lower than those in the FCA-induced arthritis group.

Over the study period, the body weight of normal mice increased steadily. The body weights of the adjuvant-injected mice (Group II) also increased, but less than that of the normal mice (Group I). As shown in Table 1, the weights of liver and spleen of the FCA-injected mice (Group II) were increased significantly compared with the normal group (Group I). However, no difference was observed in the weights of the organs belonging either to the normal group (Group I) or to the PE-treated mice groups (Groups III and IV). The administration of PE did not cause increases of AST and ALT used to assess hepatocyte integrity. The creatinine level used to assess kidney function had no significant difference in all group (Table 1). These results indicate that no liver or kidney failure was detected in the PE treated mice. Lipoproteins are macromolecules of lipids and proteins that transport lipids, including cholesterol and triglycerides, through the vascular and extravascular body fluids, and an increase in HDL-cholesterol and reduction in TG, total-cholesterol and LDL-cholesterol is considered to protect against cardiovascular diseases. In the present study, a daily intake of PE (100 mg/kg) for 3 weeks significantly decreased LDL-cholesterol and increased HDL-cholesterol compared with the FCA-treated arthritis group; effects which may be beneficial in the prevention of ischemic heart disease.

Effects of pine pollen extract on the CIA

CIA, a T cell-dependent, Ab-mediated autoimmune condition induced by type II collagen (CII), constitutes a

Table 1. Body and organ weights, AST, ALT, creatinine and lipid levels of serum after 21 days

	Group I	Group II	Group III	Group IV
Initial body weight (g)	25.6 ± 0.8367	26.4 ± 1.0954	25.6 ± 0.8367	25.6 ± 0.4472
Final body weight (g)	30.4 ± 1.0954[a]	27.6 ± 1.0954	26.6 ± 0.9747	27 ± 0.5
Liver (g)	1.152 ± 0.0756[a]	1.814 ± 0.0191	1.118 ± 0.0851[a]	0.998 ± 0.0285[a]
Kidney (g)	0.37 ± 0.0152	0.412 ± 0.0191	0.41 ± 0.0881	0.36 ± 0.095
Spleen (g)	0.124 ± 0.0075[a]	0.24 ± 0.0405	0.138 ± 0.0198[a]	0.116 ± 0.0181[a]
AST (U/mg)	358.63 ± 10.471	404.8 ± 14.6	389.24 ± 5.528	397.77 ± 20.88
ALT (U/mg)	83.342 ± 6.8273[a]	107.43 ± 3.4136	108.77 ± 2.9468	103.38 ± 2.5618
Creatinine (μg/mg)	2.7118 ± 0.2547	3.2359 ± 0.1788	2.7558 ± 0.1943	2.6366 ± 0.4469
TG (mg/dL)	145.79 ± 11.172	169.38 ± 1.9155	142.84 ± 0.2979	144.94 ± 5.9588
Total cholesterol (mg/dL)	465.15 ± 11.663	516.67 ± 31.926	466.67 ± 24.302	480.3 ± 10.74
LDL-cholesterol (mg/dL)	283.85 ± 11.045[a]	352.07 ± 25.592	281.79 ± 15.127[a]	311.48 ± 8.8747
HDL-cholesterol (mg/dL)	152.1 ± 8.8754	130.72 ± 9.2703	156.31 ± 6.7109[a]	139.84 ± 3.2057

Group I, non-immunized normal mice; Group II, FCA-induced arthritic mice not treated with sample; Group III, arthritic animals treated with pine pollen extract (100 mg/kg); Group IV, arthritic animals treated with pine pollen extract (200 mg/kg).
[a] Significantly different from Group II, $p < 0.05$.

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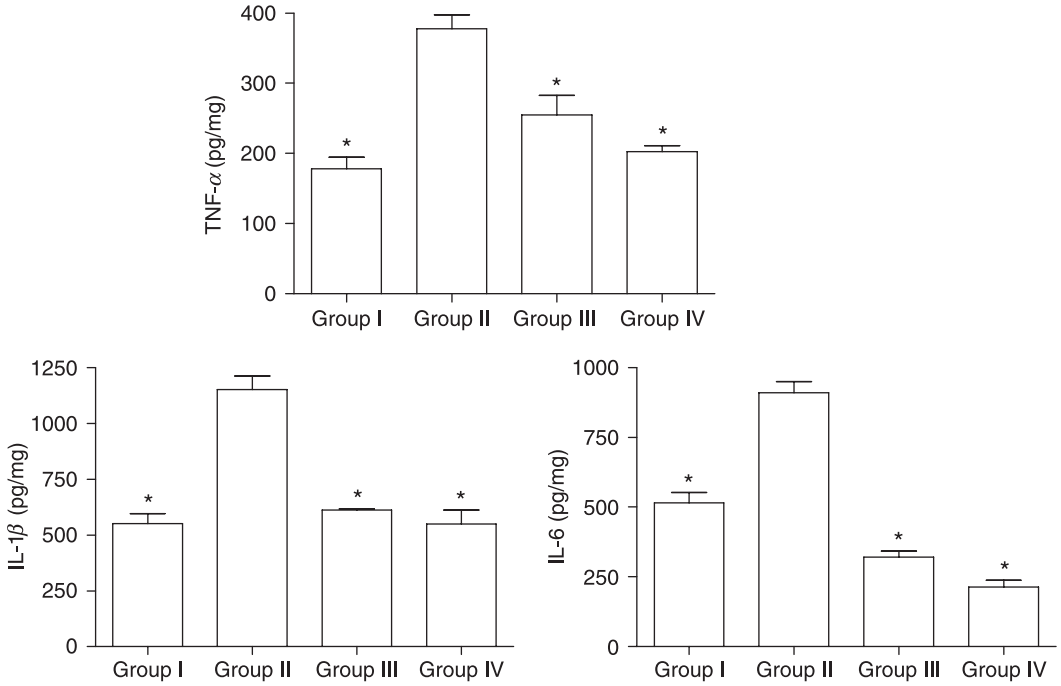

Figure 2. Effects of pine pollen extract on the FCA-induced cytokine production in serum after 21 days. Group I, non-immunized normal mice; Group II, arthritic mice not treated with sample; Group III, arthritic animals treated with pine pollen extract (100 mg/kg); Group IV, arthritic animals treated with pine pollen extract (200 mg/kg). Results are expressed as mean ± SEM ($n = 7$). * Significantly different from Group II, $p < 0.05$.

widely accepted experimental model of inflammatory joint disease, particularly rheumatoid arthritis (RA) (Myers *et al.*, 1997). A strong inflammatory cell infiltrate, proliferation of the synovial cell lining (pannus formation), and cartilage and bone destruction are seen in both CIA and RA. Proinflammatory cytokines (IL-1β, IL-6, TNF-α) and different chemokines are involved in the pathogenesis of the immune-mediated joint damage observed in CIA and RA (O'Shea *et al.*, 2002). To investigate the effect of PE on established CIA, mice were treated with a daily administration of PE (100 and 200 mg/kg) or with vehicle for 49 days. The parameters for assessment of arthritis in CIA animals (i.e. clinical score and paw swelling) were significantly changed compared with non-immunized animals throughout the experiment. The arthritic score in the PE (100 and 200 mg/kg) groups was reduced significantly ($p < 0.05$) compared with that in the CIA group (Fig. 3A). Consistent with the clinical scoring, measurements of paw swelling in this study also showed PE to be highly effective (Fig. 3B). Taken together, these results demonstrate that PE, at doses of 100 and 200 mg/kg/day, had a preventive effect on CIA. The most important point in the treatment of rheumatoid arthritis is the prevention of synovitis and pannus formation followed by cartilage damage and bone destruction (Zvaifler, 1993). It is likely that PE inhibited neutrophils from infiltrating the synovial space by preventing them from interacting with endothelial cells at the synovial site.

Rheumatoid factor (RF) was described as immunoglobulins (IgGs) in rheumatoid arthritis (Cook and Angello, 1992). The RF titer, particularly IgG RF correlates with the intensity of synovitis. RF can be found in serum samples from a variety of autoimmune diseases including rheumatoid arthritis (Cook and Angello, 1992). In the present study, oral administration of PE

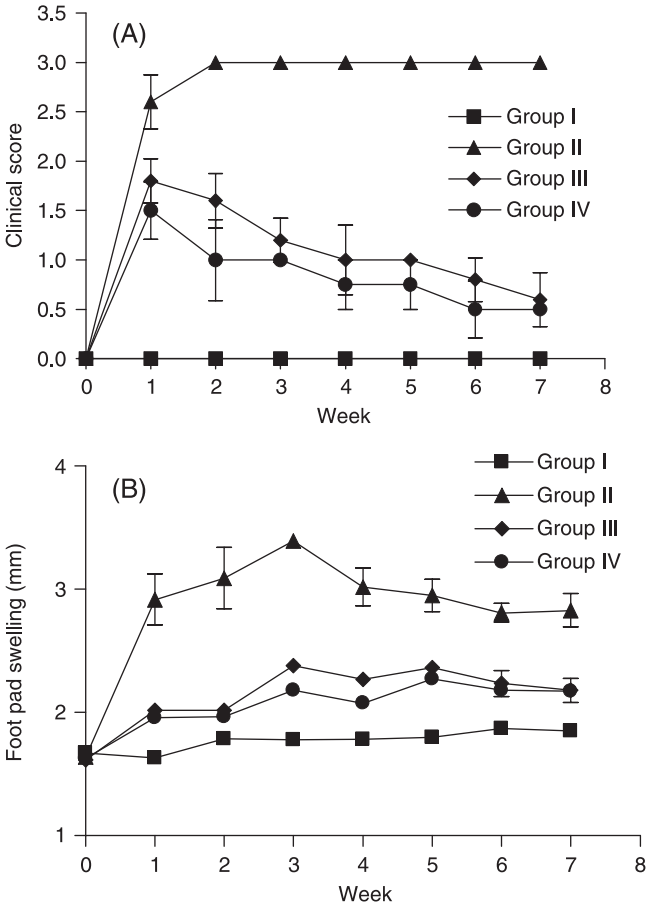

Figure 3. Progression of established CIA in DBA/1J mice. Group I, non-immunized normal mice; Group II, CIA mice; Group III, CIA mice treated with pine pollen extract (100 mg/mL); Group IV, CIA mice treated with pine pollen extract (200 mg/mL). Results are expressed as mean ± SEM ($n = 7$).

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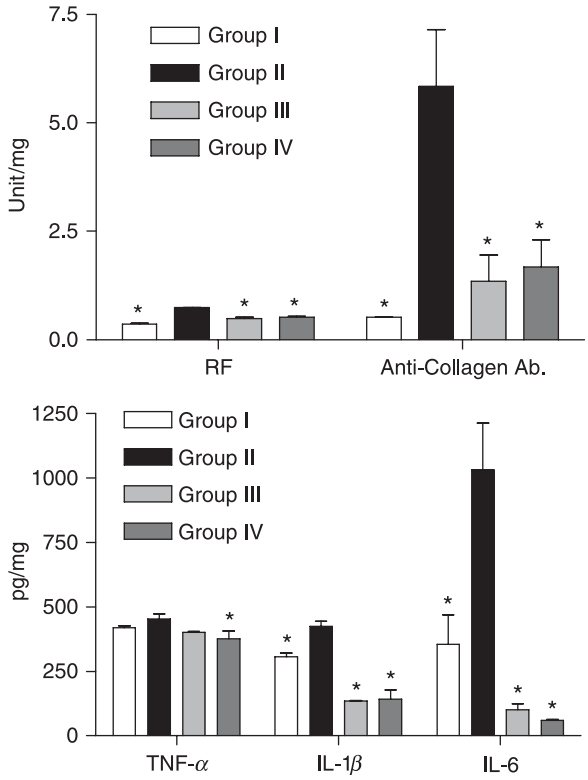

Figure 4. Levels of rheumatoid factor, anti-type II collagen antibody, cytokines in the blood of DBA/1J mouse after 49 days. Group I, non-immunized normal mice; Group II, CIA mice; Group III, CIA mice treated with pine pollen extract (100 mg/mL); Group IV, CIA mice treated with pine pollen extract (200 mg/mL). Results are expressed as mean ± SEM (n = 7). * Significantly different from Group II, $p < 0.05$.

(100 and 200 mg/kg) for 49 days reduced the formation of RF in the CIA mouse serum (Fig. 4). Type II collagen is widely used as an immunogen for the CIA model. In CIA-susceptible mice, serum antibodies to the type II collagen used for immunization are very high, and highly cross-react to various species of type II collagen. The appearance of anti-collagen antibodies in the sera of mice was followed by an ELISA for anti-collagen-IgG. In the groups treated with PE, the anti-collagen antibody level induced by type II collagen was significantly suppressed (Fig. 4). To obtain insight into the potential mechanisms involved in the amelioration of CIA by PE, the production of different cytokines (TNF-α, IL-1β, and IL-6) was investigated (Fig. 4). As expected, the serum levels of all the cytokines analysed were upregulated in arthritic mice compared with non-arthritic mice and treatment with PE for 49 days resulted in a decrease of these cytokine levels. It has been well demonstrated that TNF-α, IL-1β and IL-6 are highly expressed at sites of diseases in collagen-induced arthritis (Marinova-Mutafchieva *et al.*, 1997). These cytokines accelerate pannus formation and finally cause cartilage damage and bone destruction. Thus, the regulation of these cytokines may be important in the pathogenesis and therapy of rheumatoid arthritis (Arend and Dayer, 1995). As shown in Table 2, the administration of PE caused no increase in the AST and ALT activities and creatinine levels in serum. These results indicated no liver or kidney failure in the PE treated mice. The LDL-cholesterol of the PE (100 mg/kg) group was significantly lower than that of the CIA group. As this lipid is a dangerous factor in arteriosclerosis (Iwai *et al.*, 2002), PE may have a useful effect on lipid metabolism. This study suggests that PE may be useful in the prevention of rheumatoid arthritis owing to chronic inflammation.

The formation of α-dicarbonyl compounds is known to be an essential step for the cross-linking of proteins and subsequent free radical generation. Oxygen can accept an electron from a radical anion to form the superoxide radical anion, which can initiate damaging chain reactions (Yim *et al.*, 2001), which may contribute to lipid peroxidation and to the acceleration of the oxidative modification. During inflammation proteins can be damaged by nonenzymatic glyoxidation (Singh *et al.*, 2001). Schiff bases are formed when glucose or oxidized glucose interact with surface accessible ε-amino groups. Subsequently, Amadori rearrangements occur with the formation of ketoamine and finally to the formation of advanced glycation end-products (AGEs) (Miyata *et al.*, 1996). AGE modified proteins, unlike 'normal' proteins, can activate macrophages and stimulate the secretions of IL-1, IL-6 and TNF-α (Takagi *et al.*, 1997), which accelerate bone resorption and may participate in cartilage degradation (Isomaki and Punnonen, 1997). In the present study, levels of PCO, AGE and MDA in serum were higher in the CIA group than in the normal group after 49 days and treatment with PE (100 mg/kg) reduced serum levels of PCO, AGE and MDA versus the CIA group (Fig. 5). Several authors have suggested a role for free radicals in the pathogenesis of rheumatoid arthritis (Henrotin *et al.*, 1993). They found increased oxidized proteins and lipid peroxidation in RA compared with normals (Chen *et al.*, 1999; Sattar

Table 2. Body weight, AST, ALT, creatinine and lipid levels in DBA/1J mouse serum after 49 days

	Group I	Group II	Group III	Group IV
Initial body weight (g)	16.25 ± 0.25	15.4 ± 0.8367	15.6 ± 1.3038	15.75 ± 0.6292
Final body weight (g)	18.50 ± 0.50	17.4 ± 0.4472	18 ± 0.7071	19 ± 0.5774
AST (U/mg)	212.71 ± 32.992[c]	317.96 ± 28.952	146.85 ± 12.154[c]	267.9 ± 24.428
ALT (U/mg)	369.55 ± 26.768	397.88 ± 26.828	314.14 ± 33.377	308.05 ± 24.479
Creatinine (μg/mg)	3.6935 ± 0.2258	4.0013 ± 0.9728	2.8155 ± 0.1736	3.1004 ± 0.2577
TG (mg/dL)	136.01 ± 13.037[a]	181.18 ± 7.7349	146.77 ± 15.882	139.12 ± 15.481
Total cholesterol (mg/dL)	340.00 ± 7.7739	374.55 ± 17.794	353.64 ± 12.897	350 ± 15.581
LDL-cholesterol (mg/dL)	214.95 ± 6.0237[a]	246.85 ± 7.4974	219.7 ± 11.928[a]	223.2 ± 8.8512
HDL-cholesterol (mg/dL)	97.850 ± 2.3976	104.86 ± 4.9165	104.58 ± 3.2349	98.972 ± 3.9527

Group I, non-immunized normal mice; Group II, CIA mice; Group III, CIA mice treated with pine pollen extract (100 mg/mL); Group IV, CIA mice treated with pine pollen extract (200 mg/mL). [a] Significantly different from Group II, $p < 0.05$.

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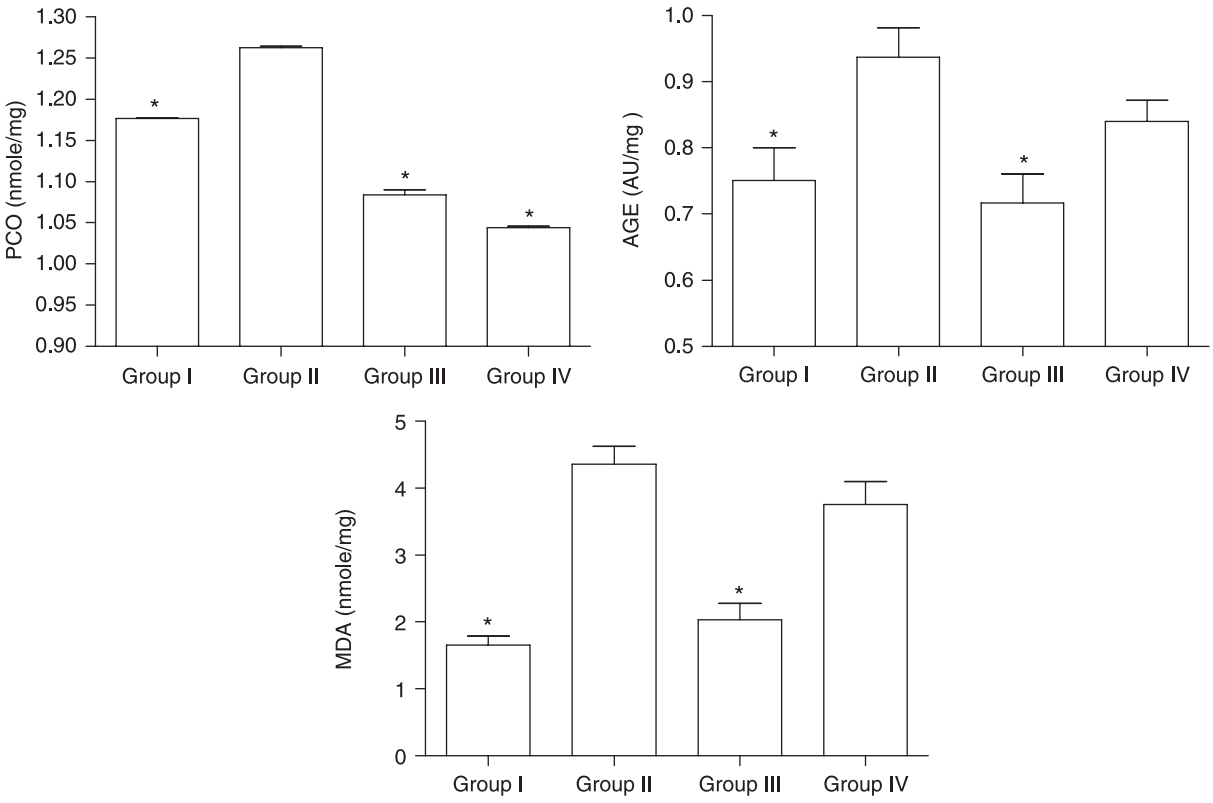

Figure 5. PCO, AGE and MDA levels in the serum of DBA/1J mouse after 49 days. Group I, non-immunized normal mice; Group II, CIA mice; Group III, CIA mice treated with pine pollen extract (100 mg/mL); Group IV, CIA mice treated with pine pollen extract (200 mg/mL). Results are expressed as mean ± SEM ($n = 7$). * Significantly different from Group II, $p < 0.05$.

et al., 2003). Elevated PCO, AGE and MDA contents in the serum in our study group of CIA, compared with normal mice, supported these reports. PE prevented accumulation of oxidized protein and lipid peroxides in serum. This provides a probable explanation of prevention of RA by PE.

In conclusion, the results of the present study clearly demonstrate that pine pollen exerts potent anti-inflammatory actions in the arthritis model and was found to be effective in chronic inflammatory conditions. Therefore, pine pollen might be proposed as a candidate for the treatment of joint inflammation. The biological mechanism of pine pollen in inflammatory diseases has not been elucidated fully, but it is hoped that the present study will contribute to the development of clinical treatments for chronic inflammatory diseases like rheumatoid arthritis.

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