□원저 : 실험연구□

# Effect of a Flavon Extracted from *Artemisia absinthium* on Collagen Induced Arthritis in Mice

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**Purpose :** In this study, a possible suppressive effect of a flavon extracted from *Artemisia absinthium* on a mouse collagen-induced arthritis (CIA) model was investigated.
**Methods :** DBA/1 mice were injected intradermally with emulsified chicken type II collagen. Three weeks after immunization, a flavon was introduced p.o. everyday. Clinical incidences of arthritis and arthritis index were measured. Measurement of anti-collagen antibodies and a stimulation index of the splenocytes of the mice were measured. IL-10 and TNF-$\alpha$ in the supernatants of the mice sera were measured by ELISA. mRNA expression for IL-10 and TNF-$\alpha$ in the splenocytes were tested.
**Results :** Flavon extracted from *Artemisia absinthium* appears to be an effective suppressor of CIA in mice. The serum anti-collagen antibody level and stimulation index of the cultured splenocytes showed no significant differences among the three experimental groups. Also serum IL-10 and TNF-$\alpha$ levels did not show any significant differences among the three experimental groups. An increased expression of mRNA for IL-10 was observed in the splenocytes treated with flavon.
**Conclusion :** With these results, flavon extracted from *Artemisia absinthium* appears to have a suppressive effect of CIA. The mechanism of the suppressive effect of flavon extracted from *Artemisia absinthium* may be from a stimulation of IL-10 production. **(Korean J Pediatr 2004;47:677-684)**

**Key Words :** Flavon, *Artemisia absinthium*, Collagen induced arthritis, IL-10, TNF-$\alpha$

## Introduction

Rheumatoid arthritis is a disease characterized by chronic idiopathic inflammation of the synovium associated with soft tissue swellings and effusions. Vascular endothelial hyperplasia with mononuclear and plasma cell infiltration is prominent. Pannus formation in advanced diseases results in subsequent erosion of the cartilage and the contiguous bone. Some symptoms such as edema, pain and limitation of motion, although not specific, suggests the disease. Juvenile rheumatoid arthritis in childhood usually presents differently, but in cases with a positive rheumatoid factor it continues into adulthood similar to rheumatoid arthritis. Despite extensive studies, the etiology is still unknown. Immunogenetic susceptibility and an external environmental trigger is considered to be necessary to cause the pathogenic inflammatory reaction in the synovium[1].

Without knowing the etiology, medications with various mechanism of action which share the activity to suppress inflammation are used. Non steroidal anti-inflammatory drugs (NSAID) are the mainstay of therapy and disease-modifying anti-rheumatoid drugs (DMARD) such as methotrexate, sulfasalazine, gold, antimalarial drugs are added. Recently, monoclonal antibodies to cytokines such as TNF-$\alpha$ and IL-1, known to play a major role in the inflammation, have shown great promise[2].

A wide variety of other biologic agents which modulate individual cell population or molecular species involved in the inflammatory process are being studied and many are in development. Drugs extracted from plants such as the polyphen fraction[3] from green tea, diterpenoid[4-6] purified from the water soluble and lipid soluble extract of *Acanthopanax Koreanum*, and taxol[7] extracted from *Taxus cu-*

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*spidata* have shown much promise in the collagen induced arthritis (CIA) model of mouse and are now being studied for in vivo use.

Continuous effort in developing new drugs for the treatment of rheumatoid arthritis paradoxically reveals that the cure is yet to be found. Novel drugs with less side effects and more effective anti-inflammatory action show great potential but are largely untested for clinical use.

Yoon[8] have recently reported on the anti-inflammatory effects of flavon extracted from *Artemisia absinithium* which inhibit the expression of inflammatory cytokines in D10S cells and chondrocytes. However, in vivo efficacy of this agent has not yet been studied.

Thus we investigated the efficacy of flavon extracted from *Artemisia absinthium* in inhibiting the inflammation and treating CIA in an animal model of rheumatoid arthritis.

## Materials and Methods

### 1. Study animals

Female DBA/1 mice 5 weeks of age were imported from Charles Liver Japan and adapted to our animal lab for 2 weeks before being put into the experiment at the age of 7 weeks (20-25 g).

### 2. Induction of CIA

Purified chicken type II collagen (Sigma, St. Louis, MO, USA) was dissolved in 0.01N acetic acid to a concentration of 2 mg/mL and mixed for 18 hours at 4℃ and reconstituted with the same amount of complete Freund's adjuvant (Sigma, St. Louis, MO, USA) to make an emulsion. One hundred μL of the mixture was intradermally injected near the proximal part of the tail of the mouse at the beginning of the experiment and 3 weeks after.

### 3. Study groups

The study animals were divided into three groups. Group 1 had collagen injected to induce arthritis and 1 mg/kg of flavon solution was given per oral for 6 days a week. Group 2 had collagen injected and intraperitoneal injection of dexamethasone was given twice a week. Group 3 had collagen injected but no other drugs were given.

### 4. Assessment of the arthritis index

Gross examination twice every week was done to check for induration, swelling, and deformity during the experiment. CIA score was graded accordingly. With no signs of induration, swelling or deformity a score of 0 was given. Minimal swelling with induration in 1-2 toes was graded as 1. Marked induration with localized swelling in many toes was graded as 2. Score 3 was given if the swelling and induration extended up to the knees and limitation of motion was detected. The sum of the total scores on all four limbs were added to a maximal score of 12. At least three people participated in the scoring to come to a consent and lessen intraobserver bias.

### 5. Evaluation of the immune reaction

#### 1) anti collagen antibody

Cardiocentesis was done to draw blood at the 10th week of experiment. Serum was separated and stored in negative 70℃ until the experiments. Thawing was done just before the ELISA test for the anti collagen antibody. Using a 96 well polystyrene microplate (Nunc, Denmark), chicken type II collagen (10 μg/mL) was melted in 0.1 M PBS (phosphate buffered saline) and kept at 4℃ for 16 hours before washing with PBS-0.05% Tween 20 four times. To prevent nonspecific binding, PBS-0.5% ovalbumin was added for 1 hour at room temperature and them washed with PBS-0.05 % Tween 20 four times. The mouse serum was diluted with PBS to 1 : 100 and reacted in the well for 1 hour and then was washed with PBS-0.05% Tween 20 four times. Peroxidase-conjugated anti-mouse IgG, A, M were added to the well for 2 hours and was reacted with 5-aminosalicylic acid. The optical density was measured at 450 nm and each sample was tested twice for the average value.

#### 2) Splenic mononuclear cell stimulation index (SI)

To test the cell mediated immune reaction, the stimulation index of splenic mononuclear cells was measured as follows. After sacrifice of the mice on the 10th week of experiment, the spleen was removed and washed in PBS several times. The splenic tissue was spliced into thin sections and homogenized with Hank's balanced salt solution (HBSS, containing 10 nM HEPES, pH 7.4) and filtered on a sterile gauze to remove contaminating tissues. The suspension was centrifuged and after removal of the supernatant, 0.015 M Tris/0.14 M $NH_4Cl$ (pH 7.4) was added to remove the red cells and washed in HBSS three times. Isolated cells were suspended in RPMI 1640-10% fetal calf serum media to a concentratin of $2\times10^6$ cell/mL and divided into sterile well plates, 100 μL per well. One hundred

μL of the collagen solution (25 μg/mL) was added and incubated in a 5% $CO_2$ incubator at 37℃ for 96 hours. Four hours before ending the incubation, 0.5 μCi ($^3$H)-thymidine was added and the cells were harvested on the cell harvester on the glass fiber filter. The amount of intracellular ($^3$H)-thymidine was measured to calculate the stimulation index (SI) as follows.

$$SI=\frac{\text{mean CPM of } (^3\text{H}) \text{ TdR in collagen stimulated culture}}{\text{mean CPM of } (^3\text{H})\text{TdR in unstimulated culture}}$$

### 6. Staining of the Tissue

After sacrificing the mice, paws were fixed in formalin and stained with hematoxilyn-eosin for microscopic examination after decalcification.

### 7. Expression of cytokines in the splenocytes

#### 1) Extraction of the total RNA

After acquiring the supernatant as described above, using the remnant cells, the total RNA was extracted with the RNeasy minikit (Quiagen Co., Hilden, Germany). The concentration of the RNA at the wavelength of 260 nm was measured with a UV-1601PC photospectometer.

#### 2) Synthesis of the first strand cDNA

Using the RNA totaling 2 μg, cDNA was made using the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Boehringer Mannheim Co., Indianapolis, IN. USA) and GeneAmp PCR system 9600 (Perkin Elmer Corp., Branchburg, NJ, USA). To confirm the production of first strand cDNA, 10 μL of the product, 20 pmole each of Taq polymerase (Takara Shuzo Co., Shiga, Japan) 0.5 U, human beta actin primers (sense primer : 5′-GTGGGGCGCCCCAGGCACCA-3′; antisenseprimer : 5′-CTCCTTAATGT CACGCACGATTTC-3′) were mixed for the PCR. PCR was done using the GeneAmp PCR system 9600. After denaturing in 94℃ for 30 seconds, 23 cycles of 30 seconds at 60℃ and 30 seconds at 72℃ was repeated and the final extension was done at 72℃ for 10 minutes. Ten μL of the product of amplication was analyzed on 1.5% agarose gel and visualized by ethidium bromide staining with electrophoresis.

#### 3) PCR of tumor necrosis factor α (TNF α) and IL-10 using the primer

Human TNF-α primer set for RT-PCR (Stratagene, La-Jolla, LA, USA) 5′-AGCACAGAAAGCATGATCCCG-3′ and 5’-CAGAGCAATGACTCCCAAAGT-3′ and human IL-10 primer set 5′-AACCAAGACCCAGACATCAAGGCG-3′ and 5′-CAGAGCCCCAGATCCGATTTTGG -3′ were used. After denaturing in 94℃ for 5 minutes 35 cycles of 5 minutes at 60℃, one minute and 30 seconds at 72℃, 45 seconds at 94℃ and 45 seconds at 60℃ was repeated and the final extension was done at 72℃ for 10 minutes. $10^5$ herpes simplex virus pre-treated with HDMEC was used as the positive control. Ten μL of the product of amplification was analyzed on 1.5% agarose gel with electrophoresis and visualized by ethidium bromide staining.

### 8. Statistics

The arthritis index, the anti collagen antibody and splenic monomuclear cell stimulation index were analyzed with the chi square test. A $P$ value of less than 0.05 was considered to be statistically significant.

## Results

### 1. Incidence of arthritis

Arthritis was detected beginning on the fourth week after the injection of type II collagen in all of the study animals. In the control group given no treatment, the incidence increased with time and all had developed arthritis by the 7th week; 30% on the fourth week, 60% on the fifth week and 100% on the 7th week (Fig. 1).

In the group treated with dexamethasone, arthritis developed on the fourth week after the injection of type II collagen with 30% having arthritis on the fifth week and no increase thereafter ($P$<0.05).

In the group treated with flavon, similar to the other groups, arthrits developed on the fourth week after the injection of type II collagen and the incidence increased to 38% on the 5th and 6th week but decreased instead on the 7th week to 10-20% ($P$<0.05). Those given flavon showed a significant inhibitory action of the regression of the arthritis which was even comparable to the dexamethasone group (Fig. 2).

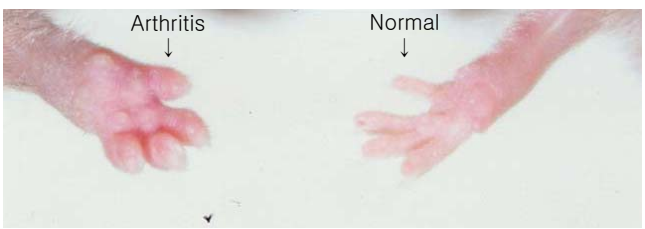


**Fig. 1.** Swollen fore foot of a DBA/1J mouse with collagen induced arthritis (right) and normal fore foot of a DBA/1J mouse treated with flavon (left).

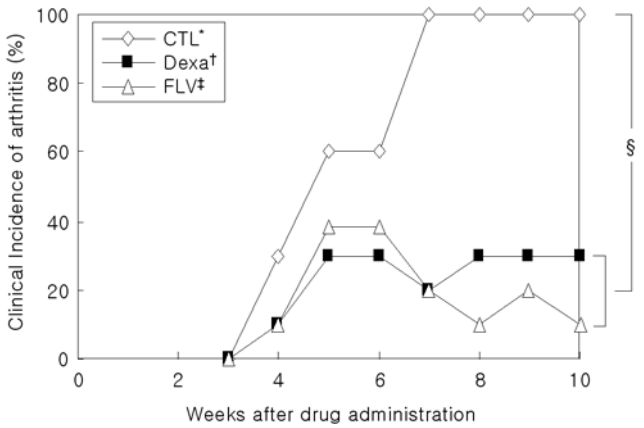


**Fig. 2.** Incidence of arthritis in each group during experiment after immunization with chicken type II collagen. The incidence of arthritis was higher in control group compared to other groups ($^{§}P<0.05$). *control, †dexamethasone, ‡flavon.

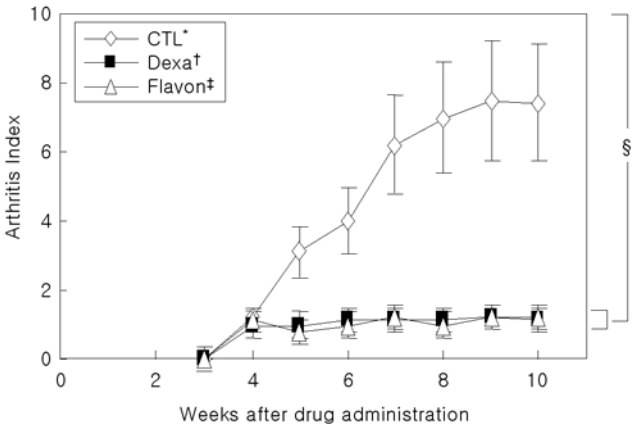


**Fig. 3.** Arthritis severity in each group during the experiment after immunization with chicken type II collagen. In flavon treated group as in dexamethasone treated group, suppression of arthritis was observed compared to control group ($^{§}P<0.01$). *control, †dexamethasone, ‡flavon.

### 2. The severity of the arthritis

The severity of the arthritis was measured with the arthritis index. The incidence of arthrits continued to increase in the control group after the injection of type II collagen; 1.2±0.3 on week 4, 3.1±0.5 on week 5, 4.0±0.8 on week 6, 6.2±1.5 on week 7, 7.5±1.7 on week 9, and 7.4±1.8 on week 10. The severity did not worsen after week 8.

The severity of arthritis with the use of flavon had decreased significantly; 1.1±0.4 on week 4, 0.8±0.3 on week 5, 1.0±0.3 on week 6, 1.0±0.4 on week 8 and 1.2±0.3 on week 10. A significant decrease in the severity of the arthritis was shown compared to the control group ($P<0.01$). In the group given dexamethasone, similar findings with the group given flavon were seen (Fig. 3).

### 3. Anti collagen antibody

The OD value of the anti collagen antibody was not significantly different in the three groups (Table 1).

### 4. Splenic mononuclear cell stimulation index

The stimulatory index of splenic mononuclear cells to collagen was not significantly different in the three groups (Table 2).

### 5. Serum levels of TNF-alpha and IL-10

The serum level of TNF-alpha and IL-10 was not different among the three groups (data not shown).

### 6. Pathologic findings of the organs

No specific pathologic change of the joint was found in the flavon and dexamethasone group but destruction of the joint capsule was seen in the control group (Fig. 4).

**Table 1.** The Levels of Anti-collagen Antibodies in each Experimental Groups

| Groups | Anti-collagen antibodies (OD) |
|---|---|
| Controls* | 0.45±0.9 |
| Dexamethasone treated* | 0.46±0.9 |
| Flavon treated* | 0.44±0.8 |

Values are mean±SD, *$P>0.05$

**Table 2.** Stimulation Indices of Splenic Mononuclear Cells to Chicken Type II Collagen in each Experimental Groups

| Groups | SI* by chicken type II collagen |
|---|---|
| Controls† | 1.9±0.9 |
| Dexamethasone treated† | 2.0±0.9 |
| Flavon treated† | 1.8±1.1 |

Values are mean±SD, *Stimulation index, †$P>0.05$

### 7. Expression of TNF-alpha and IL-10 mRNA in the splenocytes

The mRNA expression of IL-10 in the flavon group and dexamethasone group was increased but no change in the expression of TNF-alpha was found (Fig. 5).

## Discussion

Flavon had shown to be effective in the treatment of CIA in mouse. Many action mechanisms are possible but flavon is thought to induce the production of the anti in-

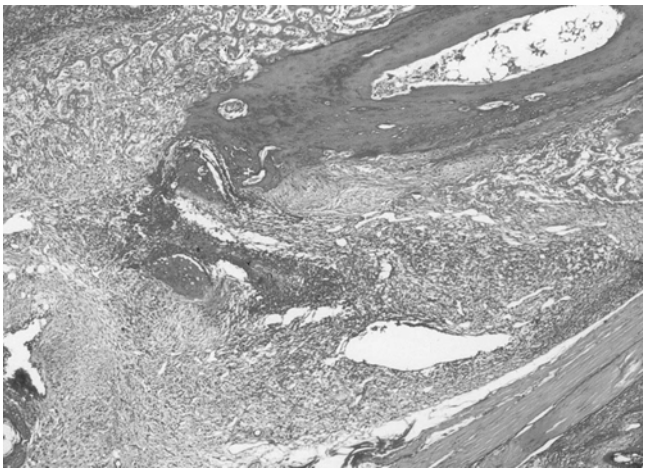

**Fig. 4.** Histology of the collagen induced arthritic foot showing destructive change of arthritic surface and marked infiltration of mononuclear cells and fibrosis in the synovium (Hematoxilyn-Eosin staining, ×40).

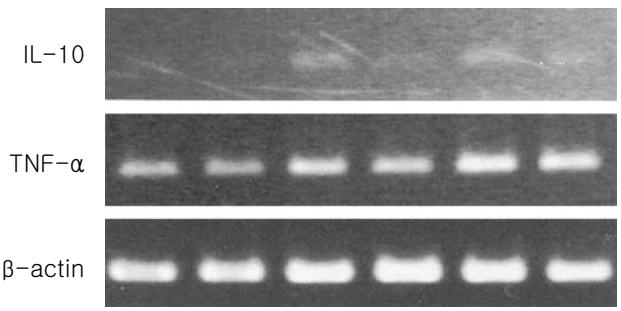


**Fig. 5.** Expression of mRNA for IL-10 and TNF-$\alpha$ in the spleen of mice (1, 2 : control mice, 3, 4 : dexamethasone treated mice, 5, 6 : flavon treated mice). Expression of mRNA for IL-10 were increased in both flavon and dexamethasone treated mice, but not in control mice). Expression of mRNA for TNF-$\alpha$ were not increased.

flammatory cytokine, IL-10 in suppressing the CIA.

Various kinds of inflammatory cytokines are involved in the chronic inflammation of the rheumatoid arthritis. TNF-$\alpha$, MIP-1$\alpha$, IL-6, IL-8, IL-18, IL-1$\beta$ are known to increase in the synovial fluid and the pro-inflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$ augment each other's action of increasing the collagenase production in chondrocytes and activation of osteoclasts in the bone. These inflammatory cytokines stimulate the endothelial cells of the high endothelial venule and increase the expression of adhesion molecules on the cell surface enhancing the migration of inflammatory cells and lymphocytes into the tissue. In additon, synovial fibroblasts stimulated with TNF-$\alpha$ and IL-1$\beta$ secrete IL-8, PGE2, and IL-6 causing acute and chronic inflammatory actions. Neutral protease and collagenase from the synovial fibroblasts

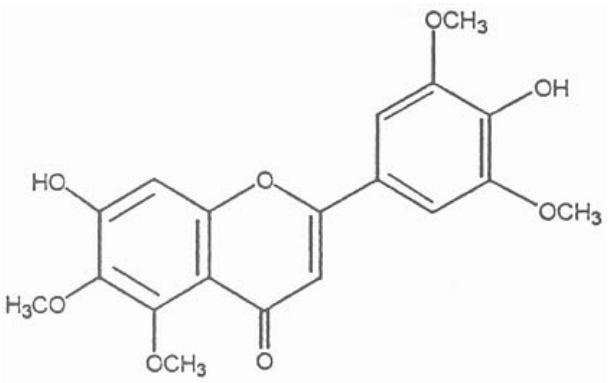


**Fig. 6.** The molecular structure of flavon extracted from *Artemisia absinthium*.

and chondrocytes destroy the collagen and proteoglycan and destroy the articular cartilage leading to arthritis[7].

Attempts to treat rheumatoid arthritis with plant derived materials are in trial and taxol has been proven to be effective in the CIS model in mice. Taxol is extracted from *Taxus cuspidata* and has been widely used as a chemotherapeutic agent in breast cancer. It is a novel antimicrotubule agent that promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cell functions. In addition, taxol induces abnormal bundles of microtubules throughout the cell cycle. In taxol treated CIA, the synoviocytes and neovascular components reverted to the naive synovial morphology with the recovery of the integrity of the intimal lining and expanded blood vessel volume[9]. These studies support the potential of taxol of being used in rheumatoid arthritis.

We have previously reported on a study with histone, a non plant derived drug with a similar action mechanism as taxol, on the CIA in the mice model. Histone stabilizes the DNA by regulating the condensation of chromatins, by folding the DNA and moving them into the cell[10]. In addition to the role of histone in the nucleic acid, other roles outside the nucleic acid and extracellular millieu are being investigated. Histone is found as an integral component in the cytoskeleton of the sea urchin egg. The axonemal microtubules in the sea urchin sperm flagella are found to be stabilized by a protein identical to histone H1[11]. The new role of histone in the assembly of microtubules is similar to the role of taxol and theoretically lays the basis of the usage in the treatment of rheumatoid arthritis. As reported

previously[12] histone effectively regresses the CIA in the mouse model and the effect is comparable to that of dexamethasone. Anti-inflammatory cytokines such as IL-10 is involved in the process.

In addition to taxol, the polyphen fraction[3] from the extract of green tea has been found to inhibit the inflammation in the CIA model of mouse and is being developed into a novel drug.

Work had been done with the water soluble portion of the extract from acanthopanax in the CIA mouse model but was found to be disappointing[5, 6]. However, with the lipid soluble portion, diterpenoid, the CIA in mouse model regressed with the increase of the anti-iflammatory cytokine IL-10. The possibility of using these data in drug research was reported[4].

Yoon[7] has recently reported on the many roles of flavon extracted from Artemisia absinthium specifically the expression of various anti-inflammatroy cytokines. It inhibited the expression of inflammatory cytokines in the cultured chondrocytes as well. Flavon extracted from *Artemisia absinthium* is a 5, 6, 3, 5, tetra methoxy 7, 4, hydroxy flavon with the structure shown in Fig. 6. It acts by suppressing the activation of IL-1 and stimulates the anti-inflammatroy cytokine IL-10. However, this was only confirmed in the cultured D10S cells and cultured synovial cells and no study was done to confirm these results in vivo.

We designed this study to see if flavon could suppress the CIA in the mouse model. Flavon extracted from *Artemisia absinthium* showed a remarkable anti-inflammatory action as expected. We also sought to see if this anti-inflammatory action was related to the production of anti collagen antibody or the nonspecific cell mediated immunity of the splenocytes and both were proved to be not related.

We then investigated on the relationship between flavon and the inflammatory cytokines involved in the pathogenesis of rheumatoid arthritis by measuring the expression of TNF-$\alpha$ which plays a major role, and IL-10 in the serum and splenocyte of the mouse. As reported in prevous studies[5, 6], the serum levels of these cytokines was not remarkable but the expression of IL-10 in the splenocytes increased significantly. with no significant change of TNF-$\alpha$.

TNF-$\alpha$ receptor antagonists have proved to be useful in the treatment of rheumatoid arthritis and has been used in the United States, including the children[13]. This reemphasizes the role of TNF-$\alpha$ in the inflammation of rheumatoid arthritis. This drug is not effective to every patient, revealing a complex mechanism in the inflammation of rheumatoid arthritis. These results can be extrapolated to the case of CIA we can conclude that other cytokines in addition to TNF-$\alpha$ is responsible for the inflammation in rheumatoid arthritis. The TNF-$\alpha$ level in mice and the expression of TNF-$\alpha$ in splenocytes was not significantly increased in our study and we conclude that the anti inflammatory action of flavonid is mediated by a mediator other than TNF-$\alpha$.

IL-10 is a cytokine synthesis-inhibitory factor secreted from the Th2 cell in mice which inhibits the secretion of cytokines in Th1 cells[14]. Clinically, IL-10 is found to be increased in autoimmune diseases such as rheumatoid arthritis, Sjogren's syndrome, and SLE compared to the healthy population[15] and furthermore, it is vastly increased in the synovium of patients with rheumatoid arthritis[16]. T lymphocytes derived from the synovium of patients with rheumatoid arthrits expressed more IL-10 compared to the T lymphocytes from the serum[17]. Based on these results, much effort has been put to utilize IL-10 in the treatment of CIA in mice[18-20].

Walmsley et al[21] and Tanaka et al[22] reported on the decrease of incidence and severity of CIA in experimental animals by injecting IL-10 and suggested the possibility of IL-10 as a treatment modality. Not only did they report on the remarkable regression of arthritis in the mouse model after adenovirus mediated transfer of the viral IL-10, but they also reported on the regression of arthritis in intra-articular injection of human IL-10 genes[18-20]. The change in the IgG1/IgG2a ratio of the anti type II collagen antibody from the stimulatioin of the B cells[23] and the suppression of the proliferation of the T cells to type II collagen were thought to be responsible for the effect[24]. The aggravation and acceleration of the disease process of the CIA after the neutralization with a anti-IL-10 antibody was reported[25]. CIA was also found to be more severe in the IL-10 deficient B10.Q mice and IL-10 was once more proved to ameliorate the disease progress in CIA[26]. To the contrary, some reported that IL-10 aggravated the arthritis and the blood level of IL-10 in mouse was not related to the incidence of CIA[27].

According to the results of this study, the inhibitory actions of flavon on CIA is not related to the production of TNF-$\alpha$ but the stimulation of the production of IL-10. An increase in the expression of the mRNA of IL-10 was observed in the splenocytes of mice. IL-10 level in the serum

of the mice injected with flavon did not increase and this might be because the increase of IL-10 is localized in arthritis and this cannot be reflected in the blood. In the CIA model, inflammatory cells stimulated with flavon migrate to the joint and increase the production of IL-10 locally, regressing CIA.

In conclusion, CIA in the mouse model can be regressed with flavon extracted from *Artemisia absinthium* and this inhibitory action sheds light to the possibility of it being used in the treatment of inflammatory diseases such as rheumatoid arthritis. Further animal studies and toxic level study is needed to investigate on this possibility.

## 국문 요약

### *Artemisia absinthium* (애엽)에서 추출한 Flavon이 마우스에서 Collagen Induced Arthritis에 미치는 영향

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**목 적** : 애엽에서 추출한 flavon이 마우스에서 유발된 CIA의 염증을 억제하는 효과를 조사하고자 하였다.

**방 법** : DBA/1 mouse에서 chicken type II collagen을 피내 주사하였고, 접종 3주 후부터 flavon을 경구투여하였다. 세 실험군에서 관절염의 발생과 관절염 지수를 측정하였으며, 항콜라겐 항체와 비장세포의 자극지수를 측정하였다. ELISA를 이용하여 IL-10과 TNF-$\alpha$의 농도를 측정하였다. 비장세포에서의 IL-10과 TNF-$\alpha$의 mRNA 표현을 조사하였다.

**결 과** : 애엽에서 추출한 flavon은 마우스의 CIA에 대한 억제 효과를 지닌다. 실험군간의 혈청내 항콜라겐항체 농도와 배양된 비장세포에서의 자극지수에는 통계적으로 의미있는 차이는 없었다. 뿐만 아니라 세 실험군간의 혈청 IL-10과 TNF-$\alpha$ 농도의 통계적으로 의미있는 차이도 관찰되지 않았다. Flavon을 투여한 마우스의 비장세포에서 IL-10의 mRNA 표현이 증가되어 있음이 관찰되었다.

**결 론** : Flavon은 마우스에 유발된 CIA의 염증을 억제하는 효과를 갖는 것을 알 수 있었으며, 이러한 효과는 flavon이 항염증성 싸이토카인인 IL-10의 분비를 증가시키기 때문으로 생각된다.

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