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원 저

Inhibitory effects of Cervi Pantotrichum Cornu herbal acupuncture on type II collagen-induced arthritis

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초 록

Type II Collagen으로 유발된 관절염에 대한 녹용약침의 억제효과에 관한 연구

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면역억제와 면역항진의 작용을 지닌 鹿茸약침의 실험쥐에서의 type II collagen(CII)으로 유발된 관절염(CIA)에 대한 효과를 연구하였다. 본 실험에서 鹿茸약침군과 생리식염수군을 대조군으로 하여 실험쥐에게 약침시술을 하였다. 鹿茸약침이 CII에 작용하는 세포반응에 대한 효과를 검정하였는데, 대조군에서는 CII유발주사 후 24일에 관절염이 관찰되었고, CIA의 정도가 점차적으로 심해졌다. 생리식염수 처리군과 비교해 24일 동안 하루에 한번 50㎍/kg 이상 용량의 鹿茸약침은 CII처리 T cell의 interleukin 2(IL-2)와 interferon-γ (IFN-γ) 생산능력을 억제했다. 또한 鹿茸약침은 CII처리 임파절과 대식세포의 tumor necrosis facter α (TNF-α)의 생산을 억제했다. 한편 CIA에 대한 약침효능의 지표는 鹿茸약침을 14일간 하루에 한번씩 처리하면서, 소의 CII로 3주 간격으로 2번 유발접종을 실시하여 검정하였다. 첫 CII 유발접종과 동시에 일일 투여량 100㎍/kg으로 14일간의 鹿茸약침이 항체형성과 CII에 대한 지연형 과민성 뿐만 아니라 관절염의 증가도 막아주었다. 鹿茸약침을 관절염 유발성 CII의 2차 접종과 동시에 시술한 결과, 관절염과 CII에 대한 면역반응을 억제하였다. 이에 저자는 CII로 유발된 관절염에 대한 鹿茸약침의 억제효과에 대하여 보고하는 바이다.

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Key words : water extract of Cervi Pantotrichum Cornu(WECPC) ; pilose antler ; Cervus korean TEMMINCK var. mantchuricus Swinhoe ; herbal acupuncture ; type II collagen-induced arthritis(CIA) ; rheumatoid arthritis (RA) ; type II collagen(C II) ; T cell

Abbreviations : WECPC, water extract of Cervi Pantotrichum Cornu ; CII, type II collagen ; CIA, type II collagen-induced arthritis ; IL-2, interleukin 2 ; INF-γ, interferon-γ ; IL-1β, interleukin-1β ; IL-6, interleukin-6 ; TNF-α, tumor necrosis factor α ; DTH, delayed-type hypersensitivity ; CPC, Cervi Pantotrichum cornu ; RA, rheumatoid arthritis ; CFA, complete Freund's adjuvant; LN, lymph node ; SRBC, sheep red blood cells ; ELISA, enzyme-linked immunosorbent assay ; OD, optical density

I. Introduction

Cervi Pantotrichum cornu(CPC) is a major animal source medicine grown in south of Korea, China, Russia, Alaska and so on. The extract from Cervi Pantotrichum Cornu by decoction, has been widely used in the treatment of some immune-related diseases, especially rheumatoid arthritis(RA) and satisfactory results are obtained.[1),2)] CPC had been reported to have effects of the antiaging action by immune activation, the inhibition of lipid peroxidation and the homeostasis maintenance.[1),2)] However, little is known about the mode of action of this herbal medication on RA.

Immunization with type II collagen(C II) is well-known to be able to induce inflammatory polyarthritis in rats and susceptible strains of mice.[3,4)] Although immune mechanisms that include both humoral and cellular immunity to C II have been implicated in the pathogenesis of the disease[5),6)], there are much evidence that anti-C II antibodies play an important role in the initiation of the disease.[7)] Since C II-induced arthritis(CIA) in rats and mice is well-known to have both clinical and histological similarities to human rheumatoid arthritis(RA)[3),4)], these models have been widely used to evaluate anti-arthritic drugs.[7),8)]

RA has been classified as several names in Korean oriental medicine. Those are Bi(痺), RoukJulPoong(歷節風), BaekHoRoukJulPoong(白虎歷節風), TongPoong(痛風) and RyuPoongSpSung arthritis(類風濕性 関節炎) etc.[9)]

It seems that RA is an autoimmune disease in the cartilage and synovial membrane. In the initiation and development of this disease, immunological and inflammatory pathways are critical, and the antigen specific T cell responses to C II are expecially important. Many investigators have tested the hypothesis that the modulation of immune responses to C II, especially the T cell mediated response, can depress the incidence and the severity of arthritis. Treatments using cytokines and anti-cytokine antibodies have been shown up-and down-regulate

the development of arthritis induced by CII and complete Freund's adjuvant(CFA) in rodents.[10]

In this paper, we have evaluated WECPC for its effectiveness on immune responses to CII in the rat CIA. In an attempt to gain further insight into the mode of action of WECPC, we also investigated effects of WECPC on the incidence and development of arthritis in rat CIA with 4 different regimens : (1) started prior to a primary immunization, (2) started on the day of a primary immunization, (3) only after a booster injection, and (4) on the established disease. The present results show that treatment with WECPC, started concurrently with either the initial or the booster immunization, can inhibit the onset and development of arthritis and the immune responses to collagen.

Ⅱ. Materials and methods

1. Materials

Rats and DBA/1J mice were purchased from KIST(Taejon, Korea). They were allowed at least 1 week to adapt to the environment(25±3 ℃, 55±5% humidity and a 12hr light/dark cycle) and were used at 7 weeks of age. CPC tablets, a water extract of CPC were purchased from Kyungju Oriental Medical Hospital, Dongguk University, Kyungju, Korea. Each tablet contained 100㎍ of the extract. For herbal acupuncture on Kihae(CV 6) to rats, randomly selected tablets were ground and suspended in normal saline at a concentration of 50㎍/10㎕.

Radiochemicals were from Amersham International Co.(Seoul, Korea). All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co.(St. Louis, MO) or Boehringer Mannheim Biochemicals (Seoul, Korea).

2. Methods

1) Arthritis induction

Type II collagen(Sigma, St. Louis, MO) extracted from bovine articular cartilage was dissolved overnight at 4℃ in 0.1㏖/ℓ acetic acid at 2.0㎎/㎖, after which the solution was emulsified in an equal volume of complete Freund's adjuvant(CFA)(Difco Laboratories, Detroit, MI, USA) in an ice-cold water bath. Arthritis was induced by an intradermal injection of 0.1㎖ of the cold emulsion into the base of the tail. Rats were boosted subcutaneously with same volume of the emulsion 21 days later. As the CFA control, 0.1㏖/ℓ acetic acid emulsified in an equal volume of CFA alone was injected to control rats using the same schedule. The onset of arthritis was considered to be present when erythema and swelling were detected in at least one joint.

2) Preparation of lymph node(LN) cell suspension

Rats were killed under ether anesthesia on day 24 after immunization with CII. Inguinal lymph nodes and macrophages were removed aseptically and pressed through a 60 gauge steel mesh to give a single cell suspension. After filtering a 200 gauge steel mesh to remove

debris and cell clumps, the dispersed cells were washed three times with RPMI-1640 medium (BRL Gibco Co., Betheda, MD) containing 10% heat-inactivated fetal calf serum(BRL Gibco), 100 U/mℓ penicillin, 100㎎/mℓ streptomycin, 2×10^{-5}M 2-mercaptoethanol(2ME) and 10mM HEPES, and resuspended in the fresh medium at a concentration of 1×10^6 viable cells/mℓ and used for lymph node(LN) cells.

3) Lymph node and macrophage cell culture and culture supernatants

To examine blastic activity of LN cells, 100 μℓ of suspension containing 1×10^5 cells was dispensed into each well of 96-well flat-bottomed microculture plates. FCS-free RPMI or 50μg/mℓ of CII was added another 100μℓ to give a total volume of 200μℓ, as described previously[11]. These mixture were incubated at 37℃ in a humidified atmosphere with 5% CO_2. After 90hr culture, 37kBq of ^{3}H-thymidine(^{3}H-TdR : specific activity 740 GBq/mmol ; Amersham Co.) was added to each well, and the plate was maintained for another 6hr. Incorporation of ^{3}H-TdR into cells was measured by a Beckman scintillation spectrometer. To prepare culture supernatants, cells were cultured in 24 well plates at a density of 1×10^6 cells in a volume of 1.0mℓ. Either 50μg/mℓ of CII or FCS-free RPMI was added in a volume of 1.0mℓ after which the plate was maintained for 48hr at 37℃ in a humidified atmosphere with 5% CO_2. Supernatants were collected after pelleting cells by centrifugation at 100 Xg for 10min and stored at -40℃ until used.

4) Cytokine assays

Cytokines of IL-1β, IL-2, IL-6. IFN-γ and TNF-α concentrations in culture supernatants were assayed using rat cytokine ELISA Test kits from R&D Systems(Funakoshi, Co., Ltd., Tokyo, Japan) or BioSource International(CA, USA). Briefly, microplates were coated with 1 μg/mℓ of anti-cytokine in 50mM carbonate buffer(pH 9.6) for overnight at 4C, and then the wells were washed 3 times with PBS-0.05% Triton X-100. After blocking with 1% BSA in PBS, samples were added to each well and incubated for 2hr at room temperature. The wells were then washed 5 times. Bound cytokines were detected by Biotinated anti-cytokine and streptoavidin-alkaline phosphatase. After washing, the freshly prepared substrate solution(p-nitrophenol phosphate tablet, Sigma Co.) was added to each well. To stop the reaction of color development, 2N-NaOH was added to each well after 20min. The optical density(OD) was measured at 405nm. The assay was performed in duplicate according to the manufacturer's recommended procedures. The results were expressed as mean±SD(pg/mℓ) of five individual rats.

5) Hemagglutination titers

Rats were injected intraperitoneally with 4×10^8 sheep red blood cells(SRBC, Sigma). Serum hemagglutination titers on day 14 were determined in U-shaped microtiter plates(Nunc) using microtechnique as described previously.[12]

6) Arthritis assessment

The clinical symptoms of arthritis in all 4 limbs were evaluated with a visual scoring system. Arthritic lesion of a scale of 0~4 : 0 = no change, 1 = swelling and erythema of the digit, 2 = mild swelling and erythema of the limb, 3 = gross swelling and erythema of the limb, 4 = gross deformity and inability to use the limb.

The arthritis score of each rat was the sum of the scores of each of the 4 limbs, the maximum score being 16. A rat that showed a score of 1 or more was regarded to be arthritic. The incidence and day of onset of arthritis were also recorded.

7) Measurement of serum anti-C II specific antibodies titer

Blood was collected individually once a week from tail vein, and the serum anti-CII antibodies titer was measured by a solid-phase enzyme-linked immunosorbent assay(ELISA). Alternatively, blood was obtained from the rats by cardiac puncture under ether anesthesia, after which serum antibody levels to CII were measured by the ELISA as described[12]. Wells of 96-well microtiter plates(No. 3912, Becton Dickinson, Oxnard, CA) were coated with 100㎕ of CII at a concentration of 25㎍/㎖ in coating buffer at pH 9.6 for 12hr at 4℃ and washed three times with washing buffer(PBS-0.05% Triton X-100).

Wells were blocked with 200㎕ of 1% bovine serum albumin(BSA) in PBS for 1hr at room temperature, and then washed five times. Aliquots of rat test serum were added to each well(100㎕/well) in duplicate, and incubated for 2hr at room temperature. After washing, 100㎕ of biotin-conjugated polyclonal goat anti-rat immunoglobulins of Ig G, Ig M, and Ig A(Tago, Burlingame, CA) was dispensed into each well, incubated for 1hr, and washed. Streptoavidin-horseradish peroxidase conjugate(Gibco, Life Techno-logies, Grand Island, NY) was added to each well at a volume of 100㎕/well. After incubation for 45min, 100㎕ of substrate solution (o-phenylenediamine dihydroch- loride)(Sigma Co) was added.

The reaction was stopped by adding 100㎕ of 4 N H_2SO_4. The optical density(OD) of absorbance at 490nm was measured with an ELI SA reader of SPEC TRAmax microplate spectrophotometer(Molecualr Devices, Sunnyvale, CA, USA).

The quantity of IgG anti-CII antibody as anti-collagen titer was expressed as ㎎/100㎖ of serum by comparison with standard curves obtained from an affinity-purified rat anti-CII antibody control. Also, the titer was expressed as the reciprocal dilution at which the OD was 50% of the maximum OD. The subtypes of anti-collagen specific antibody were analyzed by the Rat Typer Sub-isotyping Kit purchased from Bio-Rad Lab.(Richimond, CA, USA).

8) Measurement of delayed-type hypersensitivity(DTH)

DTH to CII was assessed by the rat ear skin test according to the method described by Cremer et al.[13].

The changes in ear thickness in mm at 48hr were measured after intradermal injection with

10㎍ CII dissolved in 10㎕ 50mM Tris-HCl buffer(pH 7.2). The opposite ear was injected with an equal volume of 50mM Tris-HCl buffer and served as a control.

Measurement was made with a dial thickness gauge(Ozaki Sangyou, Tokyo, Japan) and the results were expressed as the difference in thickness between collagen-and buffer-injected ears. Also, the percentage of swelling is calculated by the following formula ;

$$\frac{\text{the thickness of footpad after the boosting}}{\text{the thickness of foodpad before the boosting}} \times 100(\%)$$

9) Analytical methods

Protein contents were determined by a Protein assay kit of Bio-Rad Laboratories(Richmond, CA, USA).

10) Statistical analysis

Results were expressed as means±SE. Differences were evaluated for significance with the nonparametric Dunnett's multiple comparison test for the arthritic scores, with the Cox-Mantel test for the incidence of arthritis, and the significance of difference between those two groups were evaluated by Student's t test.

Ⅲ. Results

1. Effect of WECPC herbal acupunctured on Kihae(CV 6) on proliferation of lymph node cells from rats immunized with collagen type II

The study was designed to examine the effect of WECPC herbal acupunctured on Kihae (CV 6) on cellular immune responses to CII. Rats were treated herbal acupuncture on Kihae (CV 6) with various doses of the extracts for 14 days from the day of immunization with CII. LN cells were obtained 21 days after immunization and cultured with or without 50㎍/㎖ of CII. The re- sults are shown in <Table I>.

LN cells prepared from non-immunized rats did not respond to in vitro stimulation with CII as measured by ^{3}H-TdR incorporation. However, cells prepared from immunized rats proliferated extensively when cul- tured in the presence of CII.

Treatment of rats with WECPC at doses of 100㎍/kg and 150㎍/kg scarcely affected the proliferative response of LN cells and showed the similar levels of ^{3}H-TdR uptake to those of immunized, saline-treated rats.

2. Effect of WECPC herbal acupunctured on Kihae(CV 6) on cytokine production from lymph node cells in response to C II stimulation in vitro

Continuous herbal acupuncture(1 time per 2 days) of WECPC on Kihae(CV 6) at doses of more than 150㎍/kg dramatically inhibits the proliferative response to in vitro stimulation of CII ; the levels of ^{3}H-TdR incorporation in ex-

Table I. Effect of WECPC on Proliferative Response of Lymph Node Cells from Rats Immunized with Type II Collagen

Dose of WECPC (μg/kg)	3H-TdR incorporated (×103)	
	Medium alone	CII
0	3.3±0.32	77.4±7.7
20	3.2±0.43	76.8±9.9
50	2.9±0.13	72.2±5.7
100	3.3±0.23	24.3±2.3*
150	2.8±0.23	13.2±3.3**

Lymph node cells were obtained from rats 24 days after immunization with CII. The cells were cultured in triplicate in the presence or absence of 50μg/mℓ CII for 96hr. The proliferation of cells were assayed by addition of 1.0μCi 3H-thymidine for the final 6 hr in culture. The data was expressed as mean±SD(in counts per minute) of five individual mice.

* $p<005$, ** $p<0.001$(significant compared with control group).

perimental rats were identical to those in non-immunized controls. The effect of WECPC herbal acupunctured on Kihae(CV 6) treatment on cytokine production from LN cells in response to CII stimulation was examined in the following experiments. As shown in <Table II>, LN cells prepared from immunized rats could secrete much higher levels of T cell cytokines IL-1β, IL-2, IL-6, TNF-α and IFN-γ in response to CII stimulation. Although treatment of mice with low doses of WECPC(lesser than 100 and 150μg/kg) did not suppress the ability of LN cells to secrete T cell cytokines, herbal acu-puncture of WECPC at doses of more than 150μg/kg on Kihae(CV 6) significantly suppressed the production of those cytokines of IL-1β, IL-2, IL-6, TNF-α and IFN-γ from LN cells.

3. Effect of WECPC herbal acupunctured on Kihae(CV 6) on hemagglutination titers to sheep red blood cells(SRBC)

The next experiments were carried out to examine the effect of WECPC on antibody production. Rats were injected intraperitoneally with SRBC, and serum was obtained 14 days later to examine hemagglutination titers.

Hemagglutination titers in WECPC-treated and control rats did not differ significantly when low doses of the extracts was given to rats <Table III>. However, herbal acupuncture of WECPC on Kihae(CV 6) at doses of more than 150μg/kg/day for 14 days markedly suppressed antibody production<Table III>.

4. Effect of WECPC herbal acupunctured on Kihae(CV6) before the primary immunization with CII

Rats were herbal acupunctured on Kihae (CV6) with various doses of WECPC on days −13 and 0 relative to the primary immunization with CII. Pretreatment of rats with WE CPC could not inhibit the development of collagen arthritis even when 100μg/kg/day of the WECPC was used for pretreatment<Table IV>.

5. Effect of WECPC herbal acupunctured on Kihae (CV6) from the day of the primary immunization with CII

Table II. Effect of WECPC Herbal Acupunctured on Kihae(CV6) on Cytokine Production from Lymph Node Cells in Response to CII Stimulation *in Vitro*

Dose of WECPC (μg/kg)	IL-1β		IL-2		IL-6		TNF-α		IFN-γ	
	Medium alone	CII	Medium alone	CII	Medium alone	CII	Medium alone	CII	Medium alone	CII
0	5.9±1.5	166.5 ±19.5	1.2±0.4	576.3 ±76	126.4 ±11	503.4 ±45	1.2±0.4	876.4 ±87	2.4±0.4	965.2 ±87
20	9.2±0.9	183.8 ±29.4	4.3±0.3	545.4 ±45	142.3 ±11	475.4 ±44	4.3±0.3	934.4 ±67	3.3±0.3	1123.4 ±131
50	8.9±1.1	194.4 ±29.8	3.3±0.2	454.3 ±65	140.3 ±12	354.3 ±45	3.3±0.2	809.4 ±76	2.2±0.2	1086.4 ±133
100	9.6±1.2	87.4 ±12.4*	4.1±1.3	123.4 ±15*	129.3 ±13	122.3 ±12*	4.1±1.3	134.3 ±14*	2.4±0.2	879.5 ±98*
150	6.5±0.5	54.8 ±12.2**	2.8±0.9	38.4 ±4.3**	102.5 ±12	78.3 ±2.2**	2.8±0.9	77.5 ±8.4**	1.4±0.2	665.3 ±67**

Lymph node cells were obtained from rats 24 days after immunization with CII. Supernatants were prepared 48 hr after culture and cytokine concentration was measured by ELISA. The data was expressed as mean±SD(picogram per ml) of five individual mice.

* $p<0.05$, ** $p<0.001$(significant compared with control group).

Table III. Effect of WECPC Herbal Acupunctured on Kihae(CV6) on Hemagglutination Titers to Sheep Red Blood Cells(SRBC)

Dose of WECPC (μg/kg)	HA titer
0	10.5±1.0
20	14.4±2.2
50	13.9±1.1
100	8.7±0.4*
150	4.9±0.6**

Rats(five/group) were injected intraperitoneally with 4×108 erythrocytes on day 0. Various doses of WECPC were herbal acupunctured on Kihae(CV6) to rats for 14 days started on the day of immunization. Hemagglutination titers(HA) were measured on day 14 and the results were expressed as mean±SD.

* $p<0.05$, ** $p<0.001$(significant compared with control group).

Rats were 14 daily herbal acupunctured on Kihae(CV6) with various doses of WECPC or saline for 14 days started on the day of the primary immunization with CII. Treatment of rats with WECPC prevented the development of collagen arthritis in a dose-dependent manner <Table V>.

A satisfactory significant prevention of the disease was achieved by treating the rats with 100μg/kg/day of the WECPC herbal acupunctured on Kihae(CV 6), while no clear effects were produced by the treatment with low doses (lesser than 100μg/kg) of the drug. The effects were accompanied by the inhibition of DTH to collagen measured on day 48 and of anti-CII antibody production on days 21 and 48.

Table IV. Effect of WECPC Herbal Acupunctured on Kihae(CV 6) Treatment(Days-13 to 0) on the Development of Collagen Arthritis

	Dose of WECPC herbal acupunctured on Kihae(CV6) (μg/kg)			
	20	50	100	Saline
Incidence of arthritis	5/6	5/5	4/6	9/9
Arthritis index	12.3 ±2.2	12.5 ±1.6	11.2 ±1.6	10.5 ±1.2
Days of onset	28.5 ±2.3	31.2 ±3.2	32.6 ±3.4	33.3 ±2.3
Antibody levels (㎎/㎖)				
21 days	24.6 ±2.3	29.4 ±3.2	28.6 ±3.2	31.3 ±2.3
48 days	121.3 ±12.4	135.7 ±14.4	138.9 ±14.4	124 ±21.2
DTH (10^{-2}mm)	60.3 ±4.3	67.6 ±8.3	72.3 ±6.4	73.7 ±5.7

The results except for "arthritis incidence" were expressed as mean±SD.

Table V. Effect of WECPC Herbal Acupunctured on Kihae(CV 6) Treatment(Days 1 to 14) on the Development of Collagen Arthritis

	Dose of WECPC (μg/kg)			
	20	50	100	Saline
Incidence of arthritis	6/6	4/5	3/7*	7/7
Arthritis index	11.2 ±2.3	6.9 ±0.9*	4.4 ±0.6*	10.2 ±1.3
Days of onset	31.2 ±2.3	30.3 ±2.3	27.3 ±3.2	30.8 ±3.2
Antibody 'evels (㎎/㎖)				
21 days	20.3 ±2.4	9.7 ±0.4**	5.2 ±0.4**	23.2 ±3.2
48 days	102.4 ±11.4	43.3 ±4.5**	23.5 ±2.7**	112.3 ±11.2
DTH (10^{-2}mm)	60.9 ±5.6	47.6 ±8.6	13.3 ±1.2*	65.4 ±5.4

The results except for "arthritis incidence" were expressed as mean±SD.

* $p < 0.05$, ** $p < 0.001$ (significant compared with control group)

6. Effect of WECPC herbal acupunctured on Kihae(CV 6) from booster injection of CII

Rats were herbal acupunctured on Kihae(CV 6) with various doses of WECPC at adaily dose of 100μg/kg for 14 days from the day of a booster injection. WECPC suppressed the onset of arthritis<Table VI>.

Serum antibodies to collagen measured on day 48 in WECPC-treated rats at a daily dose of 100μg/kg were also significantly reduced, although no difference was observed in serum antibodies to CII measured on the day of a booster injection.

The data in <Table VI> also show that DTH responses to CII measured on day 48 was significantly suppressed by treatment of rats with the WECPC.

7. Effect of WECPC herbal acupuncture on Kihae(CV6) on the established diseases

Rats which were immunized with CII, developed arthritis on approximately 40 days after the primary immunization<Table IV~VI>. Therefore, therapeutic treatment with WECPC is regarded to be started from about 5 weeks after the primary immunization. Only arthritic rats were treated WECPC herbal acupuncture on Kihae(CV6) at daily doses of 100㎍/kg for 14 days started 40 days after the primary immunization. WECPC did not affect the course of the diseases. Its treatment also did not alter the immunological responses to CII in this regimen<Table VII>.

Table VI. Effect of WECPC herbal acupuncture on Kihae(CV6) Treatment(Days 22 to 35) on the Development of Collagen Arthritis

	WECPC (100㎍/kg)	Saline
Incidence of arthritis	3/7*	8/8
Arthritis index	3.4±0.9*	8.7±0.8
Days of onset	33.2±0.9	31.3±3.2
Antibody levels (㎎/㎖)		
21 days	7.7±0.9**	10.7±2.2
48 days	6.7±2.5**	112.3±17.5
DTH (10^{-2}㎜)	7.7±2.2*	56.5±6.5

Rats were herbal acupunctured on Kihae(CV6) with 100㎍/kg of WECPC. The results except for "arthritis incidence" were expressed as mean±SD.

*$p<0.05$, **$p<0.001$(significant compared with control group)

Table VII. Effect of WECPC Herbal Acupuncture Treatment(Days 40 to 53) on the Development of Collagen Arthritis

	WECPC (100㎍/kg)	Saline
No. of rats tested	8	10
Arthritis index	8.8±2.3	11.3±1.2
Days of onset	32.3±2.3	28.7±3.4
Antibody levels (㎎/㎖)		
21 days	23.6±2.4	26.7±4.7
48 days	109.6±11.4	121.5±19.8
DTH (10^{-2}㎜)	60.5±6.4	65.4±3.4

Rats were herbal acupunctured on Kihae(CV6) with 100㎍/kg of WECPC. The results were expressed as mean±SD.

IV. Discussion

WECPC is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanism by which the WECPC modify the clinical status of RA are not well understood. There is general consensus that $CD4^+$ T cells act as initiators of RA, by migrating to the affected joints, recognizing peptides derived from processed antigens, and releasing several types of cytokines.[13,14] Such

cytokines enhance the function of other cells, especially macrophages to produce pro-inflammatory cytokines including IL-1β and TNF-α.[15]

It was reported that administration of traditional medical extracts of Tripterygium wilfordii Hook f. into mice inhibited the capacity of T cells to produce IL-2, IL-4 and IFN-γ when the cells were stimulated in vitro with specific antigens.[16] They further showed its suppressive effects on collagen-induced arthritis in mice.[17] Previously, our WECPC inhibited production of IL-1β and TNF-α from macrophages in response to in vivo stimulation with bacterial liopolsaccharides when the extract was administered into mice once a day for 7 days(Kim et al., unpublished results), suggesting that the WECPC administered orally into the patients inhibit cytokine production from both T cells and macrophages and potent effects on RA. Therefore, in this study, we examined the influence of WECPC on cellular immune responses by using rat CIA, an experimental model for RA. The present results clearly demonstrated that the extract strongly inhibits T-cell activation including blastogenesis and cytokine production in response to antigenic stimulation in vitro. Furthermore, macrophage activation was also suppressed by the WECPC.

B cells are also critically important in the severity and length of the diseases.[18] Thus, we examined the influence of WECPC on B cell function. The data shown in <Table II> indicate the in vitro inhibitory action of WECPC on antibody formation when the extract was herbal acupunctured on Kihae(CV6) to rats during the course of response. It has rarely reported that medicinal herbal components prevented the development of CII-induced arthritis. In the present study, we examined the effect of the herbal acupuncture of WECPC on Kihae(CV6) on the development of CIA in rats and on immune responses to CII. We observed that the WECPC herbal acupuncture has significant reductive effects on the development of CIA in rats at dosages of 100~150μg/kg/week.

The CIA model has been studied extensively to elucidate pathogenic mechanisms relevant to RA. The excessive production of several types of cytokines including IFN-γ, IL-1β and TNF-α in the local of affected joints are also observed in these two diseases.[11),19),20)] IFN-γ is generally believed to play an important role in the pathogenesis of RA by its capacity to induce and enhance the expression of class II MHC antigens on various types of cells[21)]. IL-1β and TNF-α are also thought to be involved in cartilage destruction by stimulating the synthesis of metalloproteinase[22),23)] and by inhibiting proteoglycan synthesis[24)]. In this study, it is reasonable to speculate that WECPC inhibits immune responses, especially cytokine production and antibody formation, and helps to modify the clinical condition of the patient with RA. However, drawing the conclusion that inhibitory action of WECPC on immune responses is responsible for beneficial effect of the extract on RA, it is necessary to examine the following questions : (1) whether the immune suppressive activity of WECPC might contribute

to the therapeutic mode of action of the extract on RA, and (2) if observed, when the most effective times of starting the treatment with WECPC are. Therefore, we have used rat CIA and examined a second part of experiment to answer the questions described above. The data in <Table V> demonstrate that WECPC inhibits the development of CIA in a dose dependent manner when the agent was given prophy-lactically. This effect was accompanied by a marked suppression of antibody production and DTH skin response to CII. These results are in accord with the data obtained in several in vitro and in vivo systems, indicating that WECPC has a suppressive effect on various aspects of T cell-mediated immune responses, bossibly by interfering with helper T cells.[12]

From other studies concerning pathogenesis of CIA in animals, it has been suggested that humoral immunity to type II collagen play an important role. The anti-collagen titer in serum increased before onset of arthritis. The transfer of anti-collagen antibody obtained from arthritic mice can induce the arthritis. The acceleration of the severity of arthritis was attributed by treatments of IL-1β, TNF-α, IL-6, IL-12, and IFN-γ.[15] Thus, it seems that the suppression of cellular immune responses to CII could alter the sequential development of arthritis. We observed that the DTH response to CII was reduced by the herbal acupuncture of WECPC on Kihae(CV6). T cells were functionally inhibited in the production of IFN-γ <Table II>, suggesting that the modifications of the cellular immune responses to CII by the herbal acupuncture of WECPC on Kihae(CV6) might be resulted in the reduction of the development of CIA. In the initial phase of the development of CIA, Th1 lymphocytes and its cytokine. mainly IFN-γ, played progressively. It is known that anti-IFN-γ treatment at early phase of CIA reduced the severity of arthritis, and the injection of recombinant IFN-γ exerted a transient increase of the CIA severity in rats immunized with CII. In the present study, it is suggested that the herbal acupuncture of WECPC on Kihae(CV6) affected on some parameters such as anti-CII titer and cytokine production in the development of CIA. The production of IFN-γ from the splenocytes stimulated by CII ex vivo was suppressed by the herbal acupuncture of WECPC on Kihae(CV 6) at 10 days after CII-immunization.

On the other hand, WECPC treatment, which began concurrently with a booster injection, also significantly suppressed the development of arthritis and immune responses to collagen.

The precise mechanisms accounting for these phenomena are not clear, but similar observations were made by Asano et al.[17], who showed that delayed traditional Chinese extract treatment could suppress development of arthritis and of immunity to collagen. It is observed that WECPC is able to suppress clonal expansion of helper T cells, when it is administered intraperitoneally into rats at a single dose of 50μg/kg for 7 days(Kim et al., unpublished results). Therefore, although the mechanism(s) by which WECPC exerts suppressive effects on clonal T cell expansion is not

well understood, this regimen might theoretically lead to specific clonal depletion and result in inhibition of development of the diseases. Furthermore, it was shown that WECPC did not affect the severity of the disease nor did it alter antibody response and DTH skin reaction, when given therapeutically.

Since the clinical treatment with immunosuppressive agents such as cyclosporin A and FK-506 had a beneficial effect in patients with refractory RA25), WECPC might be a useful tool for the treatment of RA. It would be incredible if the drugs as powerful as this did not have serious toxicity, but further studies will be necessary to answer this question. The recommended dose of WECPC in the management and treatment of rat CIA will be 200㎍/kg, which is two-fifth of human therapeutic dose. However, biochemical and metabolic analysis of the constituents of WECPC have to be analysed in further delineating its mechanisms of action in arthritis.

V. Conclusion

In order to evaluate WECPC for its effectiveness on immune responses to CII in the rat CIA, rats were treated herbal acupuncture on Kihae(CV6) with various doses of WECPC(20, 50, 100, 150㎍/kg). In an attempt to gain further insight into the mode of action of WECPC, we also investigated the effects of WECPC on the incidence and development of arthritis in rat CIA with 4 different regimens : (1) started prior to a primary immunization, (2) started on the day of a primary immunization, (3) only after a booster injection, and (4) on the established disease. And then we have obtained results as follows.

1. LN cells prepared from non-immunized rats did not respond to in vitro stimulation with CII as measured by ^{3}H-TdR incorporation. However, cells prepared from immunized rats proliferated extensively when cultured in the presence of CII. Treatment of rats with WECPC at doses of 100㎍/kg and 150㎍/kg scarcely affected the proliferative response of LN cells and showed the similar levels of ^{3}H-TdR uptake to those of immunized, saline-treated rats.

2. LN cells prepared from immunized rats could secrete much higher levels of T cell cytokines IL-1β, IL-2, IL-6, TNF-α and IFN-γ in response to CII stimulation. Although treatment of mice with low doses of WECPC (lower than 100 and 150㎍/kg) did not suppress the ability of LN cells to secrete T cell cytokines, herbal acupuncture of WECPC on Kihae(CV6) at doses of more than 150㎍/kg significantly suppressed the production of those cytokines of IL-1β, IL-2, IL-6, TNF-α and IFN-γ from LN cells.

3. Hemagglutination titers in WECPC-treated and control rats did not differ significantly when low doses of the extracts was given to rats.

However, herbal acupuncture of WECPC on Kihae(CV6) at doses of more than 150μg/kg/day for 14 days markedly suppressed antibody production.

4. Pretreatment of rats with WECPC could not inhibit the development of collagen arthritis even when 100μg/kg/day of the WECPC was used for pretreatment.

5. In the case of WECPC herbal acupunctured on Kihae(CV6) from the day of the primary immunization with CII, a satisfactory significant prevention of the disease was achieved by treating the rats with 100μg/kg/day of the WECPC herbal acupunctured on Kihae(CV6), while no clear effects were produced by the treatment with low doses(lesser than 100μg/kg) of the drug. The effects were accompanied by the inhibition of DTH to collagen measured on day 48 and of anti-CII antibody production on days 21 and 48.

6. In the case of herbal acupunctured on Kihae(CV6) with various doses of WECPC at a daily dose of 100μg/kg for 14 days from the day of a booster injection, WECPC suppressed the onset of arthritis. Serum antibodies to collagen measured on day 48 in WECPC-treated rats at a daily dose of 100μg/kg were also significantly reduced. and DTH responses to CII measured on day 48 was significantly suppressed by treatment of rats with WECPC.

7. In the case of therapeutic treatment with WECPC started 40 days after the primary immunization, WECPC did not affect the course of the diseases. Its treatment also did not alter the immunological responses to CII in this regimen.

VI. References

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