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# Effect of *Ulmus davidiana Planch* herbal acupuncture Solution on proinflammatory cytokine IL-1$\beta$ and TNF-$\alpha$ production in collagen-induced arthritis of rats

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## Abstract

***Objective*** : We have evaluated UDHA into the joint 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 UDHA, we also investigated the effects of UDHA on the incidence and development of arthritis in rat CIA with 2 different regimens: (1) started prior to a primary immunization, (2) started on the day of a primary immunization.

***Methods*** : Male rats were immunized with an emulsion of 200 μg of CII and complete Freund's adjuvant (CFA). The rats were then given intraperitoneal(i.p) stimulation of Ulmus davidiana Planch herbal acupuncture(UDHA) or saline during the experiment. Lymph node cells were obtained from rats 14 days after immunization and cultured in vitro with CII. When compared with rats treated with saline as control, UDHA at doses of more than 20 μg/100 g rat once a day for 7 days inhibited the ability of inguinal lymph node cells to produce T cell cytokines interleukin-1β, tumor necrosis factor-α (TNF-α).

When rats were injected intraperitoneally with SRBC, hemaglutination titers in UD-treated and control rats did not differ significantly when low doses of UD was given to rats. However, i.p injection of UD at doses of more than 10 μg/100 g/day for 7 days slightly suppressed antibody production.

***Results*** : The present results show that treatment with UDHA can inhibit the onset and development of arthritis and the immune responses to collagen.

Conclusion: Therapeutic i.p injection with UD affect the clinical course of the disease and the immune response to CII.

***Key words*** : Rheumatoid arthritis, Ulmus davidiana Planch herbal acupuncture solution(UDHA), type II collagen(CII)-induced arthritis(CIA), Interleukin-1β(IL-1β), Tumor necrosis factor-α (TNF-α)

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# Ⅰ. Introduction

Immunization with type II collagen (CII) is well known to be able to induce inflammatory polyarthritis in rats and susceptible strains of mice. Although immune mechanisms that include both humoral and cellular immunity to CII have been implicated in the pathogenesis of the disease[1-2], there is much evidence that anti-CII antibodies play an important role in the initiation of the diseases[3-4]. Since CII-induced arthritis (CIA) in rats and mice is well-known to have both clinical and histological similarities to human RA[5], these models has been widely used to evaluate anti-arthritic drugs[3-4,6-7]. In the initiation and development of this disease, immunological and inflammatory pathways are critical, and the antigen specific T cell responses to CII are especially important. Many investigators have tested the hypothesis that the modulation of immune responses to CII, especially the T cell mediated response, can depress the incidence and the severity of arthritis. Treatment with UDHA could inhibit the onset and development of arthritis and the immune responses to collagen, but that UDHA could not change the severity when the disease was established.

Ulmus davidiana Planch is a deciduous tree which is widely distributed in korea. The barks of the stem and the root of this plant have been used in oriental traditional medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation[8-9]. UD is also known for their functions in maintaining or assisting blood circulation. With these background, UD water extract has been developed on the basis of the known function of the herb, as described in the literature of traditional Chinese and Korean medicines[10-12]. Recently, although UD has been used for protection against degeneration of cartilage and regeneration of damaged tissue.

In this paper, we have evaluated UDHA into the joint 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 UDHA, we also investigated the effects of UDHA on the incidence and development of arthritis in rat CIA with 2 different regimens: (1) started prior to a primary immunization, (2) started on the day of a primary immunization.

The present results showed that treatment with UDHA could inhibit the onset and development of arthritis and the immune responses to collagen.

# Ⅱ. Materials and methods

## 1. Materials and Animals

Sprague Dawley rats weighing 20 and 30g were used. The rats were allowed at least 1 week to adapt to the environment (25±3℃, 55±5% humidity and a 12/12 hrs light/dark cycle) and were used at 7 weeks of age. (Daehan Experimental Animals Ltd, Seoul, Korea)

Radiochemicals were from Amersham International Co. (Seoul, Korea). Media and sera for cell culture were purchased from Jeil Biotech Services (Taegu, Korea). Culture flasks and dishes were obtained from Nunc (Roskilde, Denmark). 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). Unless otherwise stated, all other chemicals were purchased from Sigma (CA, USA).

## 2. Induction of arthritis by CII collagen extracted from bovine articular cartilage

CII collagen (Sigma, St. Louis, MO) extracted from bovine articular cartilage was dissolved overnight at 4℃ in 0.1 M 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 the intradermal injection of 0.1 ㎖ of the cold emulsion into the base of the tail.

As the CFA control, 0.1 M 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.

## 3. Preparation of Inguinal lymph nodes (LN) cell suspension from rats on day 14 after immunization with CII.

Rats were killed under ether anesthesia on day 14 after immunization with CII. Inguinal lymph nodes 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/㎖ penicillin, 100 ㎎/㎖ streptomycin, 2 × 10-5 M 2-mercaptoethanol (2ME) and 10 mM HEPES, and resuspended in the fresh medium at a concentration of 1 × 106 viable cells/㎖ and used for lymph node (LN) cells.

## 4. LN cell culture and culture supernatants preparation

To examine blastic activity of LN cells, 100 ㎕ of suspension containing 1 × 105 cells was dispensed into each well of 96-well flat-bottomed microculture plates. FCS-free RPMI or 50 ㎍/㎖ of CII was added another 100 ㎕ to give a total volume of 200 ㎕, as described previously[13]. These mixture were the incubated at 37℃ in a humidified atmosphere with 5% CO2.

After 90 hrs culture, 37 kBq of 3H-thymidine (3H-TdR: specific activity 740 GBq/mmol; Amersham Co.) was added to each well, and the plate was maintained for another 6 hrs. Incorporation of 3H-TdR into cells was measured in a Beckman scintillation spectrometer.

To prepare culture supernatants, cells were cultured in 24 well plates at a density of 1 × 106 cells in a volume of 1.0 ㎖. Either 50 ㎍/㎖ of CII or FCS-free RPMI was added in a volume of 1.0 ㎖ after which the plate was maintained for 48 hrs at 37℃ in a humidified atmosphere with 5% CO2. Supernatants were collected after pelleting cells by centrifugation at 100 XG for 10 min and stored at -40℃ until used.

## 5. Assays of IL-1β and TNF-α concentrations in culture supernatants by ELISA

Cytokines of IL-1β 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 Bio Source International (CA, USA).

Briefly, microplates were coated with 1 ㎍/㎖ of anti-cytokine in 50 mM carbonate buffer (pH 9.6) for overnight at 4℃, 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 2 hrs at room temperature. The wells were then washed for 5 times. Bound cytokines were detected by Biotinated anti-cytokine and streptoavidin-alkaline phosphatase. After washing, freshly prepared substrate solution (p-nitrophenol phosphate tablet, Sigma Co., USA) was added to each well. To stop the reaction of color development, 2N-NaOH was added to each well after 20 min. Optical density(OD) was measured at 405 nm.

The assay was performed in duplicate according to the manufacturer's recommended procedures. The results were expressed as mean±SD (pg/㎖) of five individual rats.

### 6. Assay of serum hemagglutination titers

Rats were injected intraperitoneally with 4 × 108 sheep red blood cells (SRBC, Sigma, USA). Serum hemagglutination titers on day 7 were determined in U-shaped microtiter plates (Nunc) using microtechnique as described previously[14].

### 7. Analytical methods

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

### 8. Statistics

Results were expressed as means±SD.

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 the two groups were evaluated by Student's t test. P values <0.05 were considered significant.

## Ⅲ. Results

### 1. Influence of UDHA on proliferative response of lymph node cells from rats immunized with collagen type II.

The study was designed to examine the effect of UDHA on cellular immune responses to CII. Rats were treated i.p. with various doses of the extracts for 14 days from the day of immunization with CII.

LN cells were obtained 14 days after immunization and cultured with or without 50 ㎍/㎖ of CII. The results are shown in table 1. LN cells prepared from non-immunized rats did not respond to in vitro stimulation with CII as measured by 3H-TdR incorporation. However, cells prepared from immunized rats proliferated extensively when cultured in the presence of CII.

3H-TdR incorporated into the cells when medium was used with a dose of 10 ㎍/100 g rat, a value of 3.0±0.22 (× 103) was measured. However, CII group showed 64.6±7.3 (× 103) 3H-TdR incorporation into the cells. Also, 3H-TdR incorporated into the cells when medium was used with a dose of 15 ㎍/100 g rat, a value of 3.0±0.13 (× 103) was measured. However, CII group showed 64.5±5.6 (× 103) 3H-TdR incorporation into the cells.

In contrast, when 3H-TdR incorporation was measured with a dose of 20 ㎍/100 g rat and 40 ㎍/100 g rat, the values was observed to be 2.4±0.53 (× 103) and 2.0±0.21 (× 103), respectively. CII group showed 11.2±1.4 (×

103) and 8.7±0.3 (× 103) of 3H-TdR incorporation.

These results indicate that treatment of rats with UDHA at doses of 10 μg/100 g (3 μg/30 g rat) and 15 μg/100 g (5 μg/30 g rat) scarcely affected the proliferative response of LN cells and showed the similar levels of 3H-TdR uptake to that of immunized, saline-treated rats.

But Continuous i.p injection (1 time per 2 days) of UDHA at doses of more than 20 μg /100 g dramatically inhibits the proliferative response to in vitro stimulation of CII; the levels of 3H-TdR incorporation in experimental rats were identical to that in non-immunized controls. The effect of UDHA treatment on cytokine production from LN cells in response to CII stimulation was examined in the next experiments.

Table 1. Effect of UDHA on Proliferation of Lymph Node Cells from Rats Immunized with Collagen Type II.

| 3H-TdR incorporated (× 103) | Dose of UDHA (μg/100g rat) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | 20 | 40 |
| Medium (in counts/min) | 3.5±0.23 | 3.4±0.21 | 3.0±0.22 | 3.0±0.13 | 2.4±0.53 | 2.0±0.21 |
| CII (in counts/min) | 76.7±3.6 | 73.4±6.6 | 64.6±7.3 | 64.5±5.6 | 11.2±1.4 | 8.7±0.3 |

Lymph node cells were obtained from rats 14 days after immunization with CII. The cells were the cultured in triplicate in the presence or absence of 50 μg/mℓ CII for 96 hrs.
The proliferation of cells was assayed by addition of 1.0 μCi 3H-thymidine for the final 6 hrs in culture. The data was expressed as mean±SD (in counts per minute) of five individual rats. * $p< 0.05$ , ** $p< 0.01$ (significant compared to control).

## 2. Effect of UDHA on proinflammatory cytokine IL-1β and TNF-α production from lymph node cells in response to CII stimulation in vitro.

As shown in table 2, LN cells prepared from immunized rats could secrete much higher levels of T cell cytokines IL-1β in response to CII stimulation. Although treatment of rats with low doses of UDHA (5 and 10 μg /100 g) did not suppress the ability of LN cells to secrete T cell cytokines, i.p. injection of UDHA at doses of more than 15 μg/100 g significantly suppressed the production of those cytokines of IL-1β from LN cells.

At first, LN cells prepared from immmunized rats could secrete much higher levels of T cell cytokine IL-1β in response to CII stimulation. And there was no significant difference in values of each doses of medium groups.

In contrast, statistically significant differences was showned at doses of more than 15 μg/100 g in groups of CII stimulation. The intensity of suppression had a tendency to decrease in accordance with inceased amount of dose of UDHA. But although higher doses (15 and 20 μg/100 g) of UDHA was injected, the values were higher than values of medium groups.

Table 2. Effect of UDHA on IL-1β Production of Lymph Node Cells after CII Stimulation in vitro.

| IL-1β | Dose of UDHA (μg/100g) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | 20 | 40 |
| Medium (pg/mℓ) | 5.6±1.1 | 9.5±0.3 | 8.4±1.6 | 8.3±0.2 | 6.3±0.3 | 5.2±0.2 |
| CII (pg/mℓ) | 156.3±21.2 | 154.2±12.3 | 156.4±16.3 | 23.3±5.3 | 19.4±1.5 | 9.1±1.9 |

Lymph node cells were obtained from rats 14 days after immunization with CII. Supernatants were prepared 24 hrs after culture and cytokine concentration was measured by ELISA. The data was expressed as mean±SD (picogram per mℓ) of five individual rats. * $p< 0.05$, ** $p< 0.01$ (significant compared to control).

### 3. Effect of UDHA on proinflammatory cytokine TNF-α production from lymph node cells in response to CII stimulation in vitro.

As same as the effect of UDHA on proinflammatory cytokine IL-1β, the poinflammatory cytokine TNF-α production from LN cells was actively inhibited at doses of more than 15 μg/100 g. But proinflammatory cytokine TNF-α production & secretion did not suppress with low doses of UDHA 5 and 10 μg/100 g.

Also the result of suppression of poinflammatory cytokine TNF-α production was statistically significant at the doses of more than 15 μg/100 g as shown in table 3. The intensity of suppression of cytokine TNF-α production was increased in proportion of dose of UDHA.

Table 3. Effect of UDHA on TNF-α Production of Lymph Node Cells by CII Stimulation in vitro.

| TNF-α | Dose of UDHA (μg/100g) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | 20 | 40 |
| Medium (pg/mℓ) | 165.1±15 | 167.3±23 | 162.3±12 | 156.4±14 | 112.3±12 | 92.3±8.6 |
| CII (pg/mℓ) | 889.3±65 | 900.3±76 | 888.3±56 | 578.3±45 | 243.5±26 | 132.5±12 |

The data was expressed as mean±SD (picogram per mℓ) of five individual rats. * $p< 0.05$, ** $p< 0.01$ (significant compared to control).

### 4. Effect of UDHA on serum hemagglutination titers to sheep red blood cells (SRBC).

These experiments carried out to examine the effect of UDHA on antibody production.

Rats were injected intraperitoneally with SRBC, and serum was obtained 7 days later to examine hemagglutination titers. hemagglutination titers in UDHA-treated and control rats did not differ significantly when low doses of the UDHA were given to rats (table 4). However, i.p injection of UDHA at doses of more than 10 μg/100 g/day for 7 days slightly suppressed antibody production, as shown to be 8.2±0.3 HA titers. While i.p injection of UDHA at doses of more than 15-40 μg/100 g/day for 7 days markedly suppressed antibody production, as observed to be 4.5±0.3, 3.5±0.3 and 2.0±0.1 titers (table 4).

Table 4. Effect of UDHA on Hemagglutination Titers to Sheep Red Blood Cells (SRBC).

| | Dose of UDHA (μg/100g) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | 20 | 40 |
| HA titer | 11.2±2.1 | 12.3±1.4 | 8.2±0.3 | 4.5±0.3 | 3.5±0.3 | 2.0±0.1 |

Rats (six/group) were injected intraperitoneally with 4 × 108 erythrocytes on day 0. Various doses of UDHA were i.p injected into rats for 7 consecutive days staring on the day of immunization. hemagglutination titers (HA) were measured on day 7 and the results were expressed as mean±SD. * $p<0.05$, ** $p< 0.01$ (significant compared to control).

## IV. Discussion

Recently, CIA in mice or rats has been reported to be suppressed when these animals were treated with various synthetic or naturally occurring agents including Ulmus davidiana Planch (UD). UD is widely used in the chronic management and the treatment of RA, particularly, in Korea. According to published work that is well-accepted by the traditional oriental medicine community, UD was formulated to facilitate blood circulation as well as to reduce anti-inflammatory activity. The UD have been used for hundreds of years in this oriental region, and their safety and efficacy are well established through a long history of human use[15].

However, the mechanism by which the UD modify the clinical status of RA are not well understood.

The CIA model has been studied extensively to elucidate pathogenic mechanisms relevant to RA. Similarities between RA and CIA include the fact that susceptiblility is linked to specific MHC class II genes[16-17]. Also, the excessive production of several types of cytokines including IL-1β and TNF-α in the local of affected joints are also observed in these two diseases[18-20]. IL-1β and TNF-α are also thought to be involved in cartilage destruction by stimulating the synthesis of metallproteinase[21-22] and by inhibiting proteoglycan synthesis[23].

In a preliminary study(KS Kim et al., unpublished result), UD inhibited production of IL-1β and TNF-α from macrophags in response to in vivo stimulation with bacterial lipopolysaccharides when the extract was administered into rats once a day for 7 days, suggesting that the UD administered into the patients inhibit cytokine production from both T cells and macrophages, and potently effect on RA. UD treatment, which began concurrently with a booster injection, also significantly suppressed the development of arthritis and immune responses to collagen. In this study, we examined the influence of UD on cellular immune responses by using rat CIA, an experimental model for RA. The present results clearly demonstrated that the UD strongly inhibits T-cell activation including blastogenesis and cytokine production in response to antigenic stimulation in vitro.

We examined the effect of the i.p injection of UD on the development of CIA in rats and on immune responses to CII. In table 1, the i.p injection of UDHA at doses of more than 20 ㎍/100 g dramatically inhibits the proliferative response of LN cells to in vitro stimulation of CII. In table 2 and 3, i.p. injection of UDHA at doses of more than 15 ㎍/100 g significantly suppressed the production of those cytokines of IL-1β and TNF-β from LN cells. In table 4, i.p injection of UDHA at doses of more than 10 ㎍/100 g slightly suppressed antibody production. While i.p injection of UDHA at doses of more than 15-40 ㎍/100 g markedly suppressed antibody production. From the result, we observed that the UD injection has significant reductive effects on the development of CIA in rats at doses of 100-300 ㎍/100 g/week.

In our present study, it is reasonable to speculate that UD inhibits immune responses, especially cytokine production and antibody formation, and helps to modify the clinical condition of the patient with RA. The inhibitory action of UD on immune responses is responsible for beneficial effect of the UD on RA.

UD 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 UD in the management and treatment of rat CIA will be 20 ㎍/100 g, which is two-fifth of human therapeutic dose.

However, biochemical and metabolic analysis of the constituents of UD have to be analyzed in further delineating its mechanisms of action in arthritis. The results explain that UD is effective for the treatment of osteoarthritis in patients.

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