대한침구학회지 제23권 제2호(2006년 4월)
The Journal of Korean Acupuncture & Moxibustion Society

Original Article

Protective Action of Cartilage and Bone Destruction by Deer Antler Herbal-acupuncture Solution, the Pilose Antler of Cervus Korean TEMMINCK Var. *Mantchuricus Swinhoe*, on Type II Collagen-induced Arthritis in Mice

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Abstract

Objectives : The effects of water extract of deer antler herbal-acupunture solution(DHS), prepared from the pilose antler of *Cervus korea* TEMMINCK var. *mantchuricus Swinhoe* (Nokyong), a traditional immunosuppressive and immuno-activating Korean herbal- acupuncture, on collagen-induced arthritis(CIA:RA model) in mice was studied. Destruction of cartilage and bone are hallmarks of human rheumatoid arthritis, and controlling these erosive processes is the most challenging objective in the treatment of RA.

Methods : We investigated the tissue protective effects of deer antler treatment using established murine collagen-induced arthritis(CIA) as a model. Potential synergy of low dosages of anti-inflammatory glucocorticosteroids and deer antler was also evaluated.

Results : Treatment of established murine CIA with deer antler herbal-acupunture solution(DHS) (10-50μg/day) suppressed disease activity and protected agasinst cartilage and bone destruction. Although 10-50μg/day DHS had only a moderate effect on the inflammatory component of the disease activity, it strongly reduced cartilage pathology, as determined by histological examination. Serum cartilage oligomeric matrix protein(COMP) levels were significantly reduced, confirming decreased cartilage involvement. Histological analysis showed that bone destruction was prevented. DHS administration

* This work was supported by the research program of Dongguk university
· **Acceptance** : 2006. 3. 2. · **Adjustment** : 2006. 3. 18. · **Adoption** : 2006. 3. 18.
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increased serum IL-1Ra levels and reduced anticollagen type II antibody levels. Treatment with low-dose DHS(1㎍/day) was ineffective in suppressing disease score, serum COMP, or joint destruction. Synergistic suppression of both arthritis oseverity and COMP levels was noted when low-dose DHS was combined with prednisolone(0.05㎎/㎏/day), however, which in itself was not effective.

Conclusion : DHS was shown to have the inhibiting effects against IL-1α- and IL-1β-stimulated bone resorption. These results indicated that the DAS is not only highly stable and applicable to clinical uses in bone resorption, but also it will be served as a potent anti-inflammatory and anti-arthritic agents for treatment of human RA.

Key words : bone destruction, cartilage oligomeric matrix protein levels, collagen-induced arthritis, prednisolone, bone tissue cells, Rheumatoid arthritis; deer antler herbal-acupunture solution; Pilose antler of *Cervus korean* TEMMINCK var. *mantchuricus Swinhoe* (Nokyong)

Ⅰ. Introduction

In clinical practice, RA are the most commonly encountered of the many forms of degenerative joint disease, with the former characterised by localised degeneration of articular cartilage mainly in weight-bearing joints, it is a systemic inflammatory disorder characterised by inflammatory cell infiltration of proliferated synovial linings, and subsequent tissue erosion. Although increased protease activity has been implicated in the pathogenesis of RA, differences in mechanism associated with these disorders remains to be elucidated.

Although having a number of side effects, including osteoporosis and reduced adrenal function, glucocorticoids are potent and commonly used anti-inflammatory agents in human RA. Glucocorticoids downregulate proinflammatory cytokine production, such as IL-1 and TNF-α, by macrophages and monocytes via several mechanisms. One mechanism is through enhanced IκBα protein synthesis. IκBα forms inactive cytoplasmic complexes with nuclear factor-κB, which itself activates many immunoregulatory genes in response to proinflammatory c-ytokines[1-2]. Other mechanisms of action that have been reported recently[3] are downmodulation of histone acetyltransferase and upregulation of histone deacetyltransferase, which both affected messenger RNA transcription negatively.

Unossified horn or pilose antler cut from deer which belong to the Cervidae is generally termed "Nokyong" in Korea. Nokyong is one of the most famous Korean traditional medicines and has been considered to possess sexual-reinforcing and anti-aging actions. Thus, deer antler herbal- acupunture solution (DHS) has been used invigorate the kidney-yang, replenish vital essence and blood and strengthen muscle and bones in a traditional korean oriental medicine. Water extract of deer antler, prepared from the pilose antler of *Cervus korean* TEMMINCK var. *mantchuricus Swinhoe* (Nokyong), a traditional immunosuppressive and immuno-activating Korean herbal-acupuncture, have sometimes been compounded in recent Korean commercial restoratives, although little is yet known about the pharmacological effects or active ingredients. Extract from deer antler by water boiling methods, has been widely used in the treatment of some immune-related diseases, especially rheumatoid arthritis(RA) and satisfactory results are obtained[4-5]. However, little is known about the mode of action of this traditional medication on RA. RA has been classified as several different names in Korean oriental medicine. Those are Bi(痺), RoukJulPoong(歷節風), BaekHoRoukJulPoong(白虎歷節風), TongPoong(痛風) and RyuPoongSpSung(類風濕性) arthritis[6].

Normal joint function depends upon the structural integrity of the constituent cartilage and bone components, which in turn is dependent upon an equilibrium between the processes of tissue synthesis and degradation during cartilage and bone remodelling. It is generally accepted that proteolytic enzymes are involved in the catabolic aspect of normal tissue remodelling[7-8], and that altered activity of these enzymes is responsible for cartilage destruction and bone erosion associated with degenerative disorders such as and rheumatoid arthritis(RA).

Murine collagen-induced arthritis (CIA) is a widely used experimental model of arthritis. Neutralization of the monokines IL-1 and TNF-α before or during onset of arthritis arrested the development of CIA[9-10]. In accord with these findings, during onset of CIA predominantly Th1 responses towards collagen type II were found[11-12].

In the present study the effects of systemic high dose DHS therapy in established CIA were investigated. Furthermore, the potential synergy of combined prednisolone and DHS treatment were examined. We investigated the protective effect of DHS alone or in combination with prednisolone on disease activity as well as cartilage and bone destruction as determined histologically, radiologically and by serum measurements of cartilage oligomeric matrix protein(COMP). Anticollagen type II specific antibodies and serum IL-1Ra levels were assessed, in order to obtain an insight into the mechanism of action. The findings suggest that DHS treatment protects against cartilage and bone destruction, and that combined DHS with steroid drug treatment may provide a safe, anti-inflammatory and anti-destructive therapy in human RA.

II. Experiment

1. Meterials

1) Materials

Complete Freund's adjuvant and Mycobacterium tuberculosis (strain H37Ra) were obtained from Difco Laboratories (Detroit, MI, USA). Bovine serum albumin and prednisolone 21-sodium succinate (P-4153) were purchased from Sigma Chemicals (St Louis, MO, USA). Antimurine IL-1Ra antibodies (capture MAP-480, detection BAF-480) were obtained from R&D Systems (Minneapolis, MN, USA). PolyHRP-streptavidine (M2032) and Caseine colloid buffer (M2052) was from CLB (Amsterdam, The Netherlands). Recombinant murine IL-1Ra was purchased from R&D systems.

Deer antler extract tablets(D-13-23), a water extract of DHS were purchased from Kyungju Oriental Medical Hospital, Dongguk University (Kyungju, Korea) as an i.p injection grade for human. Each Tablet contained 100㎍ of the extract. For i.p. injection into rats, randomly selected tablets were ground and suspended in normal saline at a concentration of 50㎍/10㎕.

All reagents (including enzyme assay substrates) were obtained from Sigma Co. or Bachem, Bubendorf, Switzerland, and were of analytical grade where available.

2) Animals

Male DBA-1 mice on collagen preperation for CIA were purchased from KCTC, Genetic Resources center, Korea Research Institute of Bioscience and Biotechnology, (Taejon, Korea) and were reared in our specific pathogen-free mouse colony, and given food and water ad libitum. The mice were housed in filter top cages, and were given free access to water and food. The mice were immunized at the age of 10-12 weeks. They were allowed at least 1 week to adapt to the environment (25±3℃, 55±5% humidity and a 12 hrs light/dark cycle).

3) Collagen preparation

Articular cartilage was obtained from metacarpophalangeal joints of 1-2 year old cows. Bovine type II collagen was prepared

according to the method of Miller and Rhodes.[14] It was dissolved in 0.05 mol/l acetic acid(5mg/mℓ) and stored at -70°C.

2. Methods

1) Immunization

Bovine type II collagen was diluted with 0.05mol/ℓ acetic acid to a concentration of 2mg/mℓ and was emulsified in an equal volume of complete Freund's adjuvant (2mg/mℓ MT H37Ra). The mice were immunized intradermally at the base of the tail with 100μℓ emulsion (100 μg collagen). At day 21 the animals were boosted with an intra-peritoneal injection of 100μg collagen type II, diluted in phosphate-buffered saline (pH 7.4).

2) Assessment of arthritis

Mice were examined for visual appearance of arthritis in peripheral joints, and scores for severity were given (arthritis score) as previously described[10,13]. Mice were considered arthritic when significant changes in redness and/or swelling were noted in digits or in other parts of the paws. At later time points ankylosis was also included in the arthritis score. Clinical severity of arthritis was graded on a scale of 0-2 for each paw, according to changes in redness and swelling: 0, no changes; 0.5, significant; 1.0, moderate; 1.5, marked; and 2.0, maximal swelling and redness, and later on ankylosis. Arthritis score (mean± standard deviation) was expressed as cumulative value for all paws, with a maximum of eight and expressed as percentage of the initial score at the beginning of treatment.

3) Treatment of collagen-induced arthritis with DHS, prednisolone or DHS/prednisolone

To evaluate the effect of DHS, prednisolone or the combination DHS with prednisolone on established CIA, mice with CIA were selected at day 28 and divided into groups of at least 10 mice with similar arthritis scores. Thereafter, mice were treated twice a day intraperitoneally with DHS (1 or 10μg/day), prednisolone (0.05mg/kg /day), or with DHS and prednisolone(at the same doses for the noncombined regimens) for each of several days as indicated in the results.

4) Determination of interleukin-1 receptor antagonist levels

IL-1Ra was measured using enzyme-linked immunosorbent assay(ELISA). Briefly, Nunc Maxisorb ELISA plates(Nunc, Rostilde, Denmark) were coated with capture antibodies(5μg/mℓ, carbonate buffer, pH9.6, 24hrs at 4°C), and thereafter nonspecific binding sites were blocked with 1% bovine serum albumin/ phosphate-buffered saline-Tween. Standards and unknown samples were diluted in normal DBA-1 serum and incubated for 3hrs at room temperature. Biotinylated detection antibodies were added at concentrations of 0.2-0.4μg/mℓ in 0.5% bovine serum albumin in phosphate-buffered slaine-Tween for 1.5hrs at room temperature. Thereafter plates were incubated with PolyHRP(0.1μg/mℓ in 1% caseine colloid buffer) for 45min and orthophenylenediamine (0.8mg/mℓ) was used as substrate. Plates were read at 495nm .

5) Measurement of cartilage oligomeric matrix protein

At the end of the experiments, serum samples

were taken and murine cartilage oligomeric matrix protein(COMP) levels were determinated using ELISA under similar conditions as those described for the assay for human COMP[15]. The assay was modified by using rat COMP for coating the microtitre plates, the standard curve included in each plate and by using the polyclonal antiserum raised against rat COMP to detect the antibody[16-17]. A high cross-reactivity was found to murine COMP[18]. This was shown by parallel dilution curves of murine sera to the standard curve prepared with rat COMP, as well as in experiments in which a dilution of murine serum was added to the standard curve.

6) Determination of anticollagen antibodies

Antibodies against bovine type II collagen were examined by using an ELISA. Titres of total IgG, IgG1 and IgG2a were measured. Briefly, plates were coated with 10㎍ bovine type II, and thereafter nonspecific bindings sites were blocked with 0.1㏖/ℓ ethanolamin (Sigma Chemicals, USA). Serial 1 : 2 dilutions of the sera were added, followed by incubation with isotype-specific goat antimouse peroxidase (Southern Biotechnology Associates, Birmingham, AL, USA) and substrate (5-aminosalicyclic acid; Sigma Chemicals). Plates were read at 492㎚.

Serum autoantibody production against collagen was analyzed by immunoblotting and ELISA as described: 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[24]. Wells of 96-well microtiter plates (No. 3912, Becton Dickinson, Oxinard, CA) were coated with 100㎕ of CII at a ceoncentration of 25㎍/㎖ in coating buffer at pH 9.6 for 12hrs 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 9BSA) in PBS for 1 h 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 2hrs at room temperature. After washing, 100㎕ of biotin-conjugated polyclonal goat anti-rat immunoglobulins of IgG, IgM, and IgA (Tago, Burlingame, CA, USA) was dispensed into each well, incubated for 1 h, and washed. Streptoavidin-horseradish peroxidase conjugate (Gibco, Life Technologies, Grand Island, NY, USA) was added to each well at a volume of 100㎕/well. After incubation for 45 min, 100㎕ of substrate solution (o-phenylenediamine dihydrochloride) (Sigma Co. USA) was added. The reaction was stopped by adding 100㎕ of 4 N H2SO4. The optical density(OD) of absorbance at 490㎚ was measured with an ELISA reader of SPECTRAmax microplate spectrophotometer (Molecualr Devices, Sunnyvale, CA). The quantity of IgG anti-CII antibody as anti- collagen titer was expressed as mg/100ml 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 maxium 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).

Titres were expressed as means ± standard deviation dilution, which gives the half maximal value.

7) Assessment of arthritis

Mice were examined for visual appearance of arthritis in peripheral joints, and scores for severity were given (arthritis score) as previously described[10-13]. Mice were considered arthritic when significant changes in redness and/or swelling were noted in digits or in other parts of the paws. At later time points ankylosis was also included in the arthritis score. Clinical severity of arthritis was graded on a scale of 0-2 for each paw, according to changes in redness and swelling: 0, no changes; 0.5, significant; 1.0, moderate; 1.5, marked; and 2.0, maximal swelling and redness, and later on ankylosis. Arthritis score (mean± standard deviation) was expressed as cumulative value for all paws, with a maximum of eight and expressed as percentage of the initial score at the beginning of treatment.

8) Protein determination

Synovial fluid protein levels, for calculation of specific protelytic enzyme activity measurements and specific protein carbonyl measurements above, were determined by the method of Lowry et al. using bovine serum albumin as standard.

9) Analytical methods

Protein contents on protection against cartilage & bone distruction were determined by a Protein assay kit of Bio-Rad Laboratories (Richmond, CA, USA).

10) Statistical analysis

Protection against cartilage & bone distruction results were expressed as means ± SEM. statistical analysis was performed by y Student's t-test with $P<0.05$ for significance. Or, differences between experimental groups were tested using the Mann-Whitney U test, unless otherwise stated.

Ⅲ. Results

1. MTT assay of DHS on mouse calvarial osteoblast cells

The results of in vitro cytotoxicities showed that DHS has no any cytotoxicities in concentrations of 1-200 μg/ml. However, in higher concentration of the DHS, the MTT reduction observed and the degree of inhibition was increased in a dose-dependent manner from 1.0 mg/ml concentrations. In contrast, 200 μg/ml of LPS, a cytotoxic and inflammatory control reagent, showed the severe cytotoxicity on the mouse calvarial bone cells, resulting in 75% of cell death of the cells. These results indicated that the DHS are highly stable and applicable to clinical uses. However, for 10μg/ml of each DHS was used for the next experiments (Table 1, Fig.1).

Table 1. MTT Assay of DHS on Mouse Calvarial Osteoblast Cells

	DHS (μg/ml)					
	0	5.0	10.0	100	200	LPS
OD at 560nm	0.74±0.11	0.57±0.06	0.73±0.05	0.71±0.06	0.68±0.06	0.12±0.01

As a negative control, 200 μg/ml LPS gave significant inhibition of activity. Each point represents the mean± s.d. of 5 experiments from separate joints.

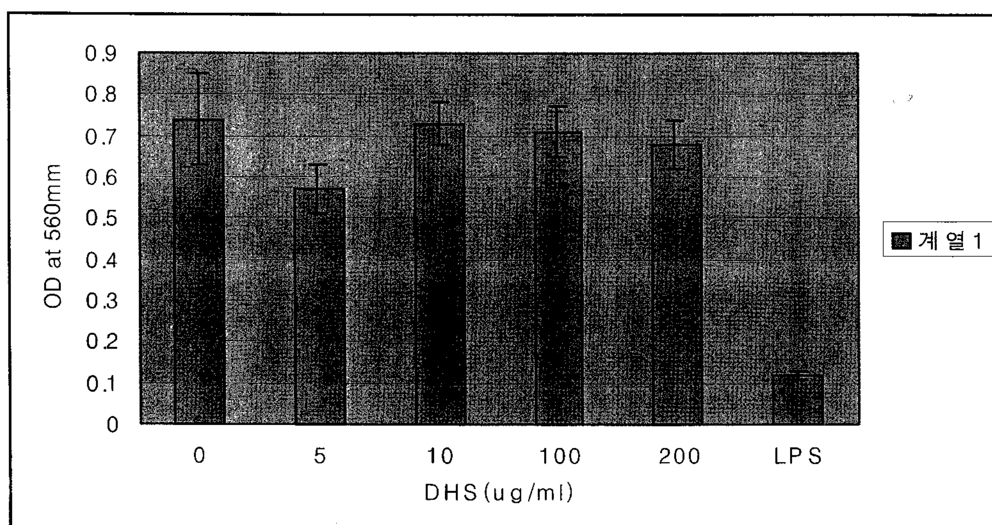


Fig. 1. MTT assay of DHS on mouse calvarial osteoblast cells

Table 2. Dose Dependent Suppression of Disease Activity of Collagen-induced Arthritis(CIA) by DHS and The Combination of DHS/Prednisolone(Pred)

Days after immunization				
	Day 24	Day 26	Day 28	Day 30
Arthritis (% of initial value) Vehicle	100	134	165	176
	114	145	176	187
	89	130	160	170
	98	125	176	178
DHS (1.0㎍)	100	133	160	170
	104	144	168	175
	100	126	148	154
	95	132	163	172
DHS (10㎍)	100	125	130	134
	104	132	138	132
	94	121	118	144
	100	123	129	123
Pred(0.5㎎/kg)	100	128	141	156
	104	126	148	154
	98	136	133	146
	97	123	142	168
DHS/Pred (10㎍ /0.5㎎)	100	107	120	125
	105	116	118	134
	103	98	123	117
	94	106	114	127

Mice with established CIA were divided into separate groups of at least 12 mice. Groups were treated intraperitoneally twice a day with vehicle, DHS, prdnisolone, or combined DHS with prednisolone for 8 consecutive days. The data represent the mean arthritis score, expressed as percentage of initial value at day 24. Experiments were repeated once with approximately the same outcome.

*$P<0.01$, versus vehicle, by Mann-Whitney U test.

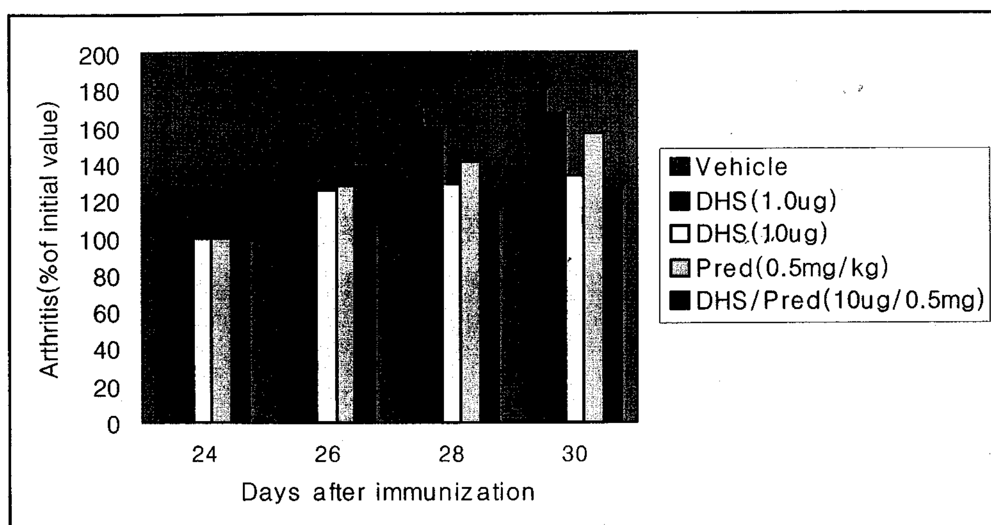


Fig. 2. Dose dependent suppression of disease activity of collagen-induced arthritis(CIA) by DHS and the combination of DHS/prednisolone(Pred)

2. Amelioration of arthritis score in collagen-induced arthritis by in vivo treatment of DHS

To investigate effects of in vivo treatment of established CIA with DHS, mice that expressed CIA at day 28 after immunization were injected intraperitoneally with vehicle, 1.0 or 10㎍ DHS per day. Table II shows that administration of 10㎍/day DHS results in significant amelioration of the arthritis score, but a lower dosage of 1.0㎍/day DHS was without effect. The anti-inflammatory effect of 10㎍/day DHS was further illustrated in Table III in which disease progression is expressed as change in (Δ) disease activity of all individual mice. Increased severity of CIA score can be seen in animals treated either with vehicle or 1.0㎍/day DHS, whereas significantly decreased disease activity was noted after treatment with 10㎍/day DHS. Histology revealed that no effect was found on the influx of inflammatory cells in joint tissues of DHS-treated animals when compared with the vehicle-treated animals. (Table 2, Fig. 2)

3. The effect of combined DHS/ rednisolone treatment

We examined potential synergistic effects of DHS and prednisolone, using low-dose prednisolone 0.05㎎/㎏/day) and 1.0 or 10㎍/day DHS. Treatment of CIA with DHS/prednisolone completely arrested the development of inflammatory signs of CIA (Table 2, 3, Fig. 2, 3). Both combinations tested revealed full suppression of disease progression. In accord with previous observations, mice treated with 0.05㎎/㎏/day prednisolone alone did not show significant suppression of arthritis. Histology taken after 7 days of treatment showed enhanced safranin O staining only in animals treated with DHS/prednisolone (10㎍ per ㎏/0.05㎏ daily), indicating reduced depletion of matrix proteoglycans (Table 4, Fig. 4). Both combinations of DHS and prednisolone reduced serum COMP to values found in naive DBA-1 mice. Interestingly, synergistic suppression of serum COMP was noted after exposure to low-dose DHS and prednisolone (Table 5, Fig. 5). In contrast to serum COMP levels, combined DHS with prednisolone treatment did not result in synergistic protection against bone destruction. High-dose DHS alone was already highly effective, and the combination of DHS with prednisolone did not improve the effect further, or was there an adverse effect of prednisolone (Table 4, Fig.4). Treatment of CIA

with 10㎍/day DHS alone and in combination with prednisolone(0.05 ㎎/㎏/day) for 7 days caused similar reduction in osteoclast numbers (data not shown).

Table 3. Dose-Dependent Arrest of Disease Activity by Treatment with DHS and DHS/Prednisolone(Pred)

Vehicle DHS(1.0㎍)	DHS (10㎍)	Pred(0.05㎎/㎏/day)	DHS/Pred(10㎍/0.5㎎)	Delta (Δ) disease activity
2.3±1.5	1.9±1.2	0.8±0.8*	1.8±1.1	0.4±0.4*

The enhanced disease activity between days 24 and 32 of each individual mouse is espressed as change in (Δ) disease activity. For treament protocol, see Fig. 2. *P<0.01, versus vehicle, by Mann-Whitney U test.

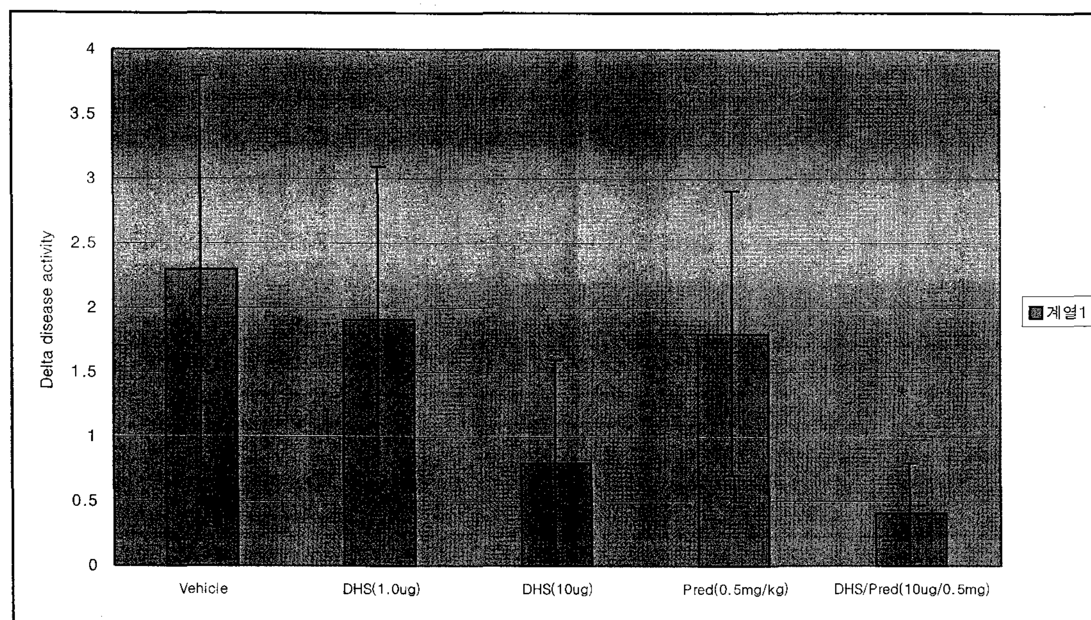


Fig. 3. Dose-dependent arrest of disease activity by treatment with DHS and DHS/prednisolone(Pred)

Table 4. The Effects of Prednisolone, DHS or DHS/Prednisolone Treatment on The Joint Pathology of Collagen-Induced Arthritis in Mice

Cartilage				Proteoglycan	Bone.	
Treatment	Dose	Infiltrate	destruction	loss	erosion	n
Vehicle	-	2.5±0.5	2.4±0.6	2.8±0.9	2.0±0.5	12
DHS	1.0	2.4±0.6	2.4±0.5	2.6±0.6	2.0±0.4	11
DHS	10	2.0±0.4	1.3±0.4*	2.0±0.4	0.7±0.1*	13
Pred	0.05	2.1±0.6	2.0±0.7	2.5±0.5	1.6±0.6	10
DHS /Pred	10/0.05	1.5±0.3*	1.2±0.7*	1.5±0.3*	0.6±0.2*	12

Histology scores of arthritic knee joints after treatment with vehicle, DHS, prdnisolone, or the combination of DHS/prednisolone. Mice were sacrified and knee joints were used for histology. Histology was scored as indicated in the Materials and Methods section. Mice were treated twice a day intraperitoneally with either prednisolone(0.05㎎/㎏), or DHS(1.0 or 10㎍/day), or DHS(1.0㎍) combined with prednisolone (0.05㎎/㎏). *P<0.01, versus vehicle, by Mann-Whitney U test.

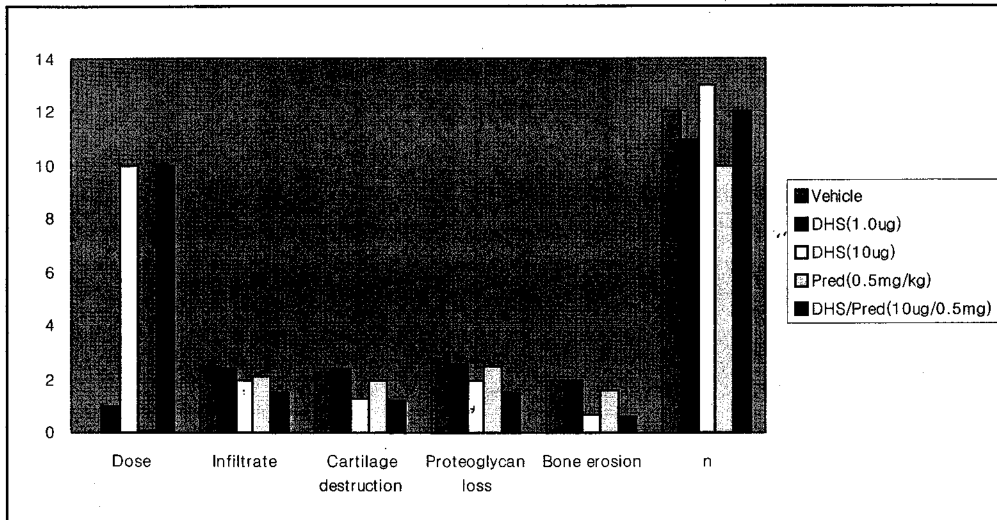


Fig. 4. The effects of prednisolone, DHS or DHS/prednisolone treatment on the joint pathology of collagen-induced arthritis in mice

Table 5. Serum Cartilage Oligomeric Matrix Protein (COMP) Level as a Marker of Cartilage Turnover

Days after immunization				
Vehicle DHS(1.0μg)	DHS (10μg)	Pred(0.05mg/kg/day)	DHS/Pred(10μg/0.5mg)	COMP levels (ug/ml)
10±2.3	7.3±1.7	4.2±0.5*	9.5±2.1	3.6±0.5*

Suppression of serum COMP was found after treatment with DHS and DHS/prednisolone (Pred). DHS(10μg/day) and DHS/prednisolone (10μg/0.05mg) reduced serum COMP levels to basic levels as found in nonimmunized animals (4.3±0.3μg/mℓ). The data represent the mean ± standard deviation COMP levels of at least five sera per group.

*P<0.01, versus vehicle, by Mann-Whitney U test.

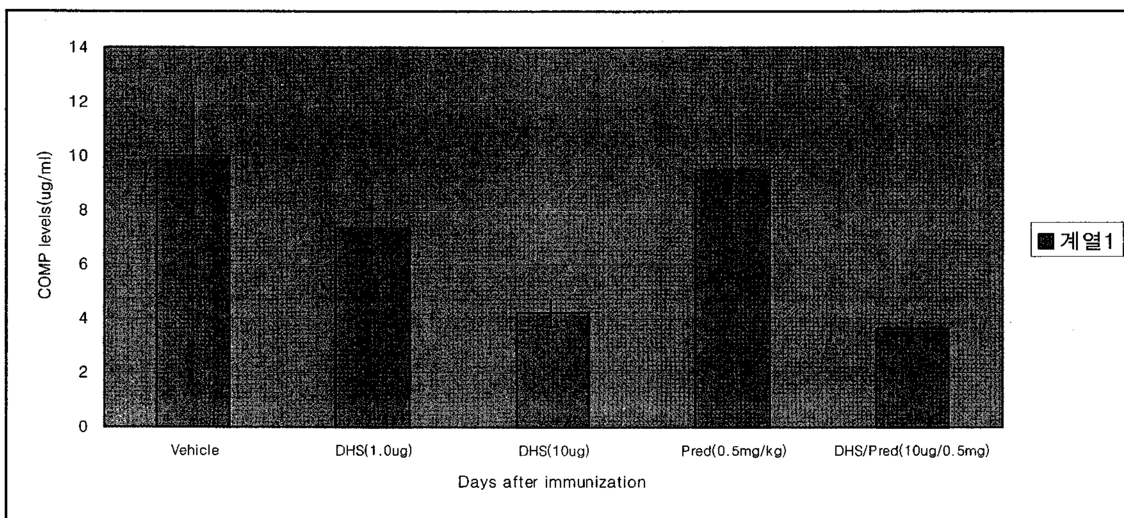


Fig. 5. Serum cartilage oligomeric matrix protein (COMP) level as a marker of cartilage turnover

Table 6. DHS or DHS/Prednisolone(Pred) Treatment is Associated with Reduced Anticollagen Type II(CII) Antibody Levels

	Vehicle	DHS(1.0㎍)	DHS(10㎍)	Pred(0.05㎎/kg)	DHS/Pred(10㎍/0.5㎎)
Anti-CII titres					
Ig tot	1920	2130	1240	1980	970
	1980	2160	1310	2050	1050
	1870	2110	1170	1930	902
	1910	2100	1211	1900	978
IgG1	1320	1180	620	1330	580
	1350	1240	680	1390	650
	1270	1200	560	1260	520
	1330	1105	610	1310	560
IgG2a	1480	1520	580	1170	480
	1540	1620	640	1230	540
	1430	1460	530	1120	420
	1495	1500	550	1178	490

Treatment with 1㎍/day DHS resulted in lower anticollagen type II antibodies. Total immunoglobulins (Ig tot), IgG1 and IgG2a levels were reduced. Similar effects were found after treatment with DHS/prednisolone (10㎍/day/ 0.05㎎/kg). Anticollagen type II were determined in at least seven mice per group. The data represent the mean ± standard deviation dilution, which gives the half maximal value.

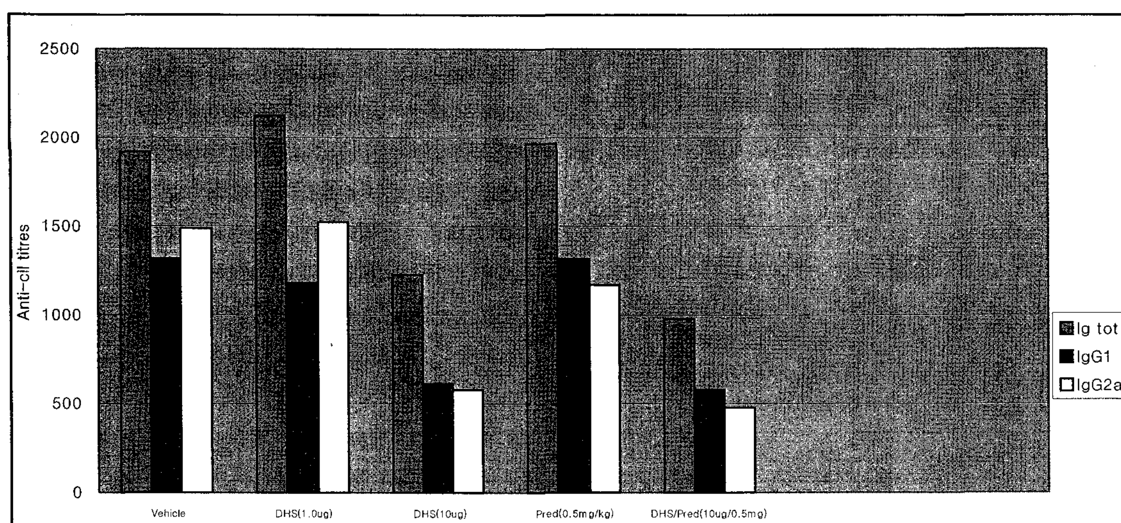


Fig. 6. DHS or DHS/prednisolone(Pred) treatment is associated with reduced anticollagen type II(CII) antibody levels.

4. The effect of DHS protects against cartilage destruction

Systemic treatment with high-dose DHS(10㎍/day) significantly decreased cartilage destruction, determined as chondrocyte death and cartilage erosions (Fig. 4) It did not result in a significantly reduced loss of matrix proteoglycans, as determined by safranin O staining (Table 4, Fig. 4). It has been demonstrated that there is a

strong correlation between severe cartilage damage and increased serum COMP levels during murine CIA. In naive DBA-1 mice, serum COMP levels are approximately 4.0㎍/㎖ and COMP levels increased up to 8-12 ㎍/㎖ in mice with fully established CIA. Serum COMP levels were determined in the various groups to identify the protection against severe cartilage destruction by DHS. (Table 5, Fig. 5) shows that elevated COMP in CIA were not reduced by treatment with low-dose DHS. It is of particular interest, that treatment with high-dose DHS(10㎍/day) significantly reduced serum COMP levels to values found in nonarthritic control animals. Bone destruction, which is a common feature of murine collagen arthritis, was examined by radiological analysis. Radiographs of knee joints were taken at the end of the treatment period. Histological analysis of knee joints corroborated the protective effect of DHS.

5. The effects of DHS, or DHS/ prednisolone treatment on interleukin-1 receptor antagonist and anticollagen antibody levels

Anticollagen antibodies were assayed at the end of treatment period at day 35. The antibody levels increased rapidly after clinical expression of CIA around day 28 after immunization. After DHS(10/㎍day) treatment for 7 days, total IgGs levels as well as IgG1 and IgG2a anticollagen type II antibody levels were lower compared with vehicle treated animals(Table 6, Fig. 6) Although all anticollagen type II antibodies were reduced, IgG2a levels showed the most prominent reduction, indicating an effect on the Th1 rather than on the Th2 immune response. No decreased anticollagen type II antibody levels were found after treatment with low-dose DHS. The high-dose DHS/prednisolone regimen reduced anticollagen type II antibodies to levels similar to those found after treatment with 10㎍/day DHS.(Table 6, Fig. 6)

Serum IL-1Ra levels were determined at the end of the experiments and (Table VII, Fig. 7) shows a twofold increase after DHS treatment (10 ㎍/day). Treatment with 1.0㎍/day DHS showed no significant effects on serum IL-1Ra levels. Prednisolone reduced IL-1Ra levels when compared with vehicle-treated animals. In accord with these findings, combined DHS with prednisolone (10㎍ per day/0.05㎎ per kg per day) treatment resulted in lower IL-1Ra levels than found with DHS alone.(Table 7, Fig. 7)

Table 7. Serum Interleukin-1 Receptor Antagonist(IL-1Ra levels) After Treatment with either DHS, Prednisolone or DHS/Prednisolone(Pred)

Treatment	Dose	IL-Ra (pg/ml)
Vehicle	-	387±98
DHS	1.0	421±112
DHS	10	765±176*
Pred	0.05	332±78
DHS/Pred	10/0.05	421±132

Serum IL-1Ra was determined using enzyme-linked immunosorbent assay at day 32 after immunization. Mice were treated as indicated in Table 1. The data represent the mean ± standard deviation of at least six mice per group. The sensitivity of the IL-1Ra assay was to within 165 pg/ml.
*P<0.01, versus vehicle, by Mann-Whitney U test.

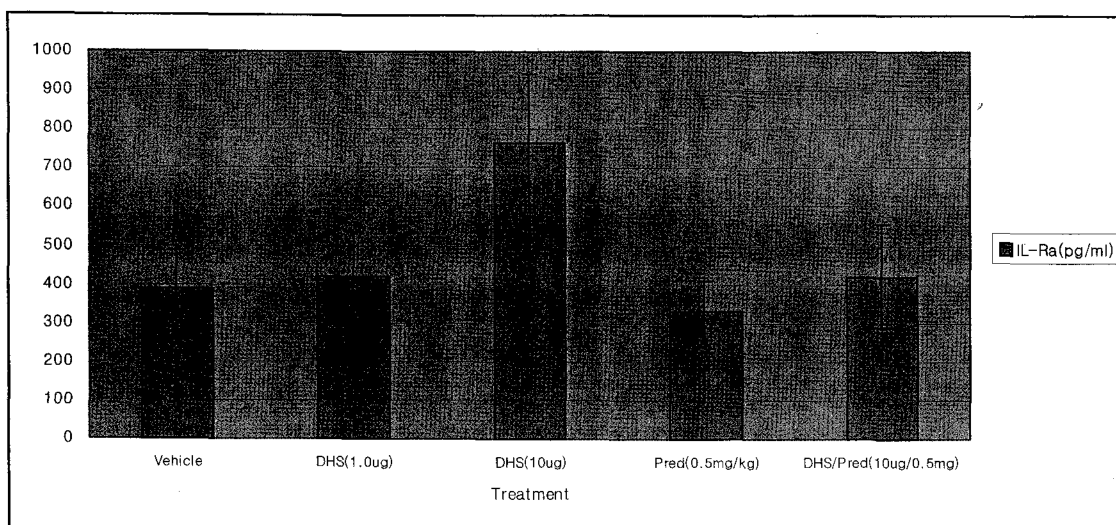


Fig. 7. Serum Interleukin-1 Receptor Antagonist(IL-1Ra levels) after treatment with either DHS, prednisolone, or DHS/prednisolone(Pred)

6. Histology

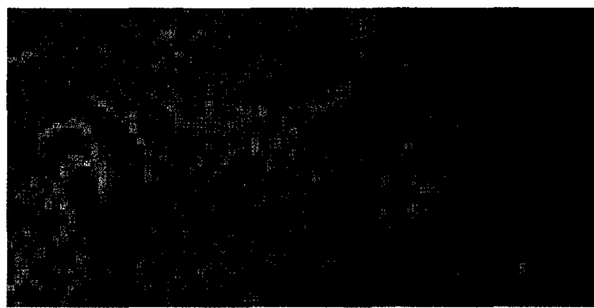
Mice were killed by ether anaesthesia. Knee joints were removed and fixed for 4 days in 4% formaldehyde. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding[10,15] Tissue sections (7μm thick) were stained with haematoxylin and eosin, or safranin O. Histopathological changes were scored using the following parameters.

Infiltration of cells was scored on a scale from 0 to 3, depending on the amount of inflammatory cells in the synovial tissues. Inflammatory cells in the joint cavity were graded on a scale from 0 to 3 and expressed as exudate. Cartilage proteoglycan depletion was determined using safranin O staining. The loss of proteoglycans was scored on a scale from 0 to 3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. A characteristic parameter in CIA is the progressive loss of articular cartilage. This destruction was separately graded on a scale from 0 to 3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of the articular cartilage. Bone erosion was scored on a scale ranging from 0 to 3, ranging from no abnormalities to complete loss of cortical and trabecular bone of the femoral head and patella. Histopathological changes in the knee joints were scored in the patella/femur region on 5 semiserial sections of the joint, spaced 70 μm apart. Scoring was performed on decoded slides by two observers, as described earlier[10,15].

All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2), and prepared for histologic examination. The sections were stained with hematoxylin and eosin. Histological grading of the inflammatory lesions was done according to the method proposed by White and Casarett[24]. These slides were scored by three independent, pathologists in a blinded manner.

(A) Normal

(B) collagen-induced arthritis



(C) arthritis+treatment of DHS

Ⅳ. Discussion & Conclusion

Deer antler herbal acupuncture solution(DHS) is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanism by which the DHS extract modify the clinical status of RA are not well understood.

Previously, our DHS 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 mice once a day for 7 days[20], suggesting that the DHS ectract 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 DHS on cellular immune responses by using rat CIA (collagen induced arthritis), an experimental model for RA.

When we examined the effects of DHS on MTT reduction in mouse calvarial cells. Whereas the LDH release assay is an index of membrane damage, the MTT reduction assay reflects intracellular redox state. Thus, inhibition of MTT is not necessarily accompanied by complete cell lysis. The results of in vitro cytotoxicities showed that DHS has no any cytotoxicities in concentrations of 1-200 μg/ml and furthermore there is no any cytotoxicity even in concentration of 30μg/ml on mouse calvarial bone cells. However, in higher concentration of the DHS, the MTT reduction observed and the degree of inhibition was increased in a dose-dependent manner from 1.0 mg/ml concentrations. These results indicated that the DAS are highly stable and applicable to clinical uses. However, for 10μg/ml of each DHS was used for the next experiments

Collagen-induced arthritis(CIA) is a widely used model of arthritis that displays several features of human RA. Recently it was demonstrated that the onset of CIA is under stringent control of DHS[2]. Furthermore it was demonstrated that exposure to DHS during the immunization stage reduced onset and severity of CIA. Several animal studies indicated that DHS administration, starting just after immunization with the disease-inducing agent, ameliorated Th1-mediated mode DHS treatment disease expression increased to control values. ls of autoimmune diseases such as experimental arthritis[21]. Expression of CIA is also under particularly stringent control by DHS[20]. However, after cessation of DHS treatment disease expression increased to control values.

Rheumatoid arthritis(RA) is associated with an increased production of a range of cytokines including tumour necrosis factor(TNF)-α and interleukin(IL)-1, which display potent proinflammatory actions that are thought to contribute to the pathogenesis of the disease[22-23]. Although TNF-α seems to be the major cytokine involved in the inflammatory process, IL-1 is the key mediator with regard to cartilage and bone destruction[16].

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 DHS extract. It was observed that the DHS injection has significant reductive effects on the development of CIA in rats at dosages of 100-150 μg/kg/week. DHS treatment also suppressed the production of the proteases of cytoplasmic, lysosomal and matrix protease types[21].

DHS extract might be a useful tool for the treatment of RA. It would be incredible if the drugs as powerful as this did not have seriuos toxicity, but further studies will be necessary to answer this question. However, biochemical and metabolic analysis of the constituents of DHS ectract have to be analysed in further delineating its mechanisms of action in arthritis.

The present study demonstrates clear tissue-protective effects of DHS, although DHS did not prove to be a very potent anti-inflammatory cytokine. Both cartilage and bone erosion were prevented by DHS treatment of established CIA. Combination with low-dose prednisolone enhanced the anti-inflammatory capacity of DHS. This might offer an attractive alternative to the use of high-dose prednisolone, because it can circumvent the unwanted side effects of the drug, including steroid-induced osteoporosis.

In previous studies of murine collagen arthritis[10,13] it was shown that TNF-α is important at onset of the disease, whereas IL-1 is the dominant cytokine, not only at the onset, but also in the progression of the arthritis and the concomitant cartilage destruction. Further support for the critical role of IL-1 is provided by the absence of collagen arthritis in IL-1β-deficient mice, and the marked reduction of this arthritis in ICE-deficient mice as well as in normal mice treated with IL-1 β-converting enzyme inhibitors[24-25]. Moreover, reduced onset of arthritis was noted in TNF-receptor-deficient mice, but once a joint was afflicted the arthritis progressed to full-blown expression and cartilage destruction, again emphasizing that TNF is important in onset, but is not the dominant cytokine in progression and tissue destruction[26].

Treatment of murine CIA with high-dose DHS (10μg/day) during the immunization stage delays onset as well as reduces severity. When DHS administration was terminated, however, disease expression and activity rapidly accelerated and was indistinguishable from that in the vehicle-treated control group[20]. DHS treatment of streptococcal cell wall arthritis in rats resulted in suppression of disease activity, and ameliorated the chronic destructive process leading to decreased lesions[21]. This was associated with enhanced levels of IL-1Ra, the natural inhibitor of IL-1, which is in accord with observations in the present study. However, it is not likely that the twofold increment in serum IL-1Ra levels, found after DHS exposure, is sufficient to suppress CIA. As previously mentioned, blockade of IL-1 by anti-IL-1 antibodies or very high-dose IL-1Ra completely suppressed CIA and lead to full protection against joint pathology[10,13].

Analysis of anticollagen type II antibodies revealed that DHS treatment did not alter the balance of IgG2a/IgG1 antibodies, indicating no suppressive effect on the Th1 immune response. Total anticollagen type II antibody levels were lower in both DHS (10 μg/day) and DHS/prednisolone treated animals when compared with the vehicle group. We have previously found that anticollagen type II antibody levels rapidly increased after onset of CIA and reached the highest levels after 7 days. DHS treatment arrested the development of high anticollagen type II antibody levels after onset and did not alter IgG2a/IgG1 balance.

Cartilage alterations were screened for by histology as well as COMP levels in sera of mice at the end of the experiments. COMP is a prominent component of articular cartilage. In a process affecting cartilage turnover, fragments are released and eventually reach the circulation. Thus, serum levels may be used as a marker of

generalized cartilage turnover[19,27]. More recent studies[28-29] have demonstrated the production of COMP by activated synovial cells and synovial tissue of RA and osteoarthritis patients. Although the relative contribution to serum levels is not firmly established, important information has been obtained from studies of collagen arthritis in rats. Thus, increased serum COMP levels are seen at time points when erosive changes appear in cartilage, whereas in early stages with marked inflammation in the synovium no increased COMP levels are seen[16-17]. Furthermore, serum COMP levels are reduced to normal in murine CIA after treatment with IL-1-blocking antibodies, in correspondence with a marked suppression of the cartilage lesion as viewed histologically[23]. Thus, evidence so far indicates that changes in serum COMP relate to changes in the cartilage turnover In accord with these findings, low-dose DHS/ prednisolone treatment did not suppress disease activity, largely reflecting synovitis, but clearly reduced serum COMP levels[30-31].

Glucocorticoids are potent and commonly accepted anti-inflammatory agents, but the major drawback on continued usage in arthritis is the severe negative effect on the bone. More recent studies on the mechanism of action revealed strong downregulation of macrophage production of the proinflammatory cytokines TNF-α and IL-1, related to enhanced IκBα synthesis. Intriguingly, over a large dose range steroids not only inhibit TNF and IL-1, but also reduce the production of IL-1Ra and regulatory cytokines such as IL-4 and IL-10[32]. This suggests that the net effect in joint inflammation is impaired by the lack of the protective cytokines, which inhibit TNF/IL-1 production as well as induce potent upregulators of scavengers such as soluble receptors for TNF and IL-1, and IL-1Ra[33]. Moreover, DHS powerfully reduces inducible nitric oxide synthase expression, thereby counteracting the suppressive effect of IL-1 on chondrocyte proteoglycan synthesis, which is mainly nitric oxide mediated. Evidence for the latter was provided in in vitro studies with nitric oxide inhibitors.

The present data clearly demonstrates the synergistic effect of combination therapy of low-dose prednisolone and DHS. Low-dose DHS was without suppressive effect on clinical disease activity. However, when combined with prednisolone the progression of CIA was completely arrested. Furthermore, synergistic suppression of cartilage destruction was demonstrated by lowered serum COMP levels, which was also reflected by histology. Only combined therapy with high-dose DHS and prednisolone was able to suppress the influx of inflammatory cells in joint tissues and reduce the loss of matrix proteoglycans.

In conclusion, DHS might offer an alternative cartilage-and bone-protective therapy that is complementary to TNF/IL-1 inhibitors. Its limited effect on the inflammatory process warrants combination with other therapeutic modalities. The present data suggest that combination with prednisolone at low dosages provides an intriguing option. It must be considered that a cocktail of DHS and low-dose glucocorticosteroids or glucocorticoids might be an even more efficacious therapy for human RA.

V. References

1. Scheiman, RI, Cogswell, PC, Lofquist, AK, & Baldwin, AS : Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 1995, 270 : 283-286.
2. Auphan, N, Didonato, JA, Rosette, C, Helmberg, A, & Karin, M : Immunosuppression by glucocorticoids : Inhibition of NF-κ B activity through induction of IκB synthesis. Science 1995, 270 : 286-290.
3. Barnes, PJ : Anti-inflammatory actions of glucocorticoids : molecular mechanisms. Clin Sci Colch 1998. 94 : 557-578.

4. T. and Nomura Y. Inhibition of lipid peroxidation ny deer antler Rokujo) extract in vivo and in vitro. Journal of Medical and Pharmaceutical Society for WAKAN-YAKU 5, 1988 ; 123-128.
5. Wang B-X, Zhao X-H., Qi S-B, Kaneko S., Hattori M., Namba T. and Nomura Y. Effects of repeated administration of deer antler (Rokujo) extract on biochemical changes related to aging in senescence-accelerated mice. Chem. Pharm. Bull. 36, 1988 ; 2587-2592 .
6. Jun-Hyouk Jang, Kap-Sung Kim : IL-1β suppresive effects of Phellodendri Cortex and Clematidis Radix aqua-acupuncture in lipopolysaccaride induced arthritis(in Korean). Dongguk University. 1-60, 1996.
7. V. Testa, F. Capasso, N. Muffulli, A. Sgambato and P.R.J. Ames, Proteases and antiproteases in cartilage homeostasis. *Clin Orthop Relat Res* 308 1994 ; 79-84.
8. T.A. Einhorn and R.J. Majeska, Neutral proteases in regenerating bone. *Clin Orthop* 262, 1991 ; 286-297.
9. Williams, RO, Feldmann, M, & Maini, RN : Antitumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Prod Natl Acad Sci USA 1992, 89 : 9784-9788.
10. Joosten, LAB, Helsen, MMA, Van de Loo, FAJ, & van den Berg, WB : Anticytokine treatment of established collagen type II arthritis in DBA/1 mice : a comparative study using anti-TNF alpha, anti-IL-1 alpha, beta and IL-1Ra. Arthritis Rheum 1996, 39 : 797-809.
11. Mauri, C, Williams, RO, Walmsley, M, & Feldmann, M : Relationship between Th1/Th2 cytokine pattern and the arthritogenic response in collagen-induced rthritis. Eur J Immunol 1996, 26 : 1511-1518.
12. Doncardi, A, Stasiuk, LM, Fournier, C, & Abehsira-Amar, O : Conversion in vivo from an early dominant Th0/Th1 response to a Th2 phenotype during the development of collagen-induced arthritis. Eur J Immunol 1997, 27 : 1451-1458.
13. Van de Berg, WB, Joosten, LAB, Helsen, MMA, & van de Loo, FAJ : Amelioration of established murine collagen-induced arthritis with anti-IL-1 treatment. Clin Exp Immunol 1994, 95 : 237-243.
14. Miller, EJ & Rhodes, RK : Preparation and characterization of the different types of collagen. Methods Enzymol 1982, 2 : 33-65.
15. Saxne, T & Heinegard, D : Cartilage oligomeric matrix protein : a novel marker of artilage turnover detectable in synovial fluid and blood. Br J Rheumatol 1992, 31 : 583-591.
16. Larsson, E, Mussener, A, Heinegard, D, Klareskog, L, & Saxne, T : Increased serum levels of cartilage oligomeric matrix protein and bone sialoprotein in rats with collagen arthritis. Br J Rheumatol 1997, 36 : 1258-1261.
17. Vingsbo-Lundberg, C, Saxne, T, Olsson, H, & Holmdahl, R : Increased serum levels of cartilage oligomeric matrix protein in chronic erosive arthritis in rats. Arthritis Rheum 1998, 41 : 544-550.
18. Joosten, LAB, Helsen, MMA, Saxne, T, et al. : Synergistic protection against cartilage destruction by low dose prenisolone and interleukin-10 in established murine collagen arthritis. Inflamm Res 1999, 48 : 48-55.
19. Mansson, B, Carey, D, Alini, M, et al. : Cartilage and bone metabolism in rheumatoid arthritis. J Clin Invest 1995, 95 : 1071-1077.
20. Jin-Gyu Kim, Kap-Sung Kim : Inhibitory effects of Cervi Pantotrichum cornu herbal cupuncture on type II collagen-induced arthritis. Dongguk University, Thesis of Master Degree. Kyungju, Korea. 2001.
21. Sang-Dong Park. Kyung-Ho Kim, Kap-Sung Kim : Inhibitory effect of deer antler aqua-acupunture, the pilose antler of *Cervus korean* TEMMINCK var. *mantchuricus Swinhoe* (Nokyong), on protease activities, antioxidant and free radical damages in synovial fluid

from rheumatis arthritis rats. Dongguk University, Thesis of Master Degree, Kyungju, Korea. 2001.

22. Arend, WP & Dayer, JM : Inhibition of the production and effects of interleukin-1 and tumor necrosis factor α in rheumatoid arthritis. Arthritis Rheum 1995, 38 : 151-160.
23. Bucala, R, Ritchlin, C, Winchester, R, & Cerami, A : Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. J Exp Med 1991, 173 : 569-574. 24. White, S.C., and Casarett, G.W. 1974. Induction of experimental autoallergic sialadenitis. J. Immunol. 112 : 178-185.
24. Christen, A, Mudgett, JS, Orevillo, CJ, et al. : Collagen-induced arthritis in the interleukin- 1 β knock-out mouse [abstract]. Trans Orthoped Res Soc 1996, 21 : 169.
25. Ku, G, Faust, T, Lauffer, LL, Livingston, DJ, & Harding, MW : IL-1β converting enzyme inhibition blocks progression of type II collagen-induced arthritis in mice. Cytokine 1996, 8 : 377-386.
26. Sandborg, C, Imfeld, KL, Zaldivar, F, Wang, Z, Buckingham, BA, & Berman, MA : IL-4 expression in human T cells is selectively inhibited by IL-1α and IL-1β. J Immunol 1995, 155 : 5206-5212.
27. Di Cesare, PE, Carlson, CS, Stollerman, ES, et al. : Expression of cartilage oligomeric matrix protein by human synovium. FEBS Lett 1997, 412 : 249-252.
28. Di Cesare, PE, Fang, C, Leslie, MP, et al. : Localization and expression of cartilage oligomeric matrix protein by human rheumatoid and osteoarthritic synovium and cartilage. J Orthop Res 1999, 19 : 437-445.
29. Mori, L, Iselin, S, De Libero, G, & Lesslauer, W : Attenuation of collagen-induced arthritis in 55 kD TNF receptor type I (TNFRI)-IgG1 treated and TNFRI deficient mice. J Immunol 1996, 157 : 3178-3185.
30. Van Meurs, JBJ, van Lent, PLEM, Singer, II, et al. : Interleukin-1 receptor ntagonist prevents expression of the etalloproteinase-generated neoepitope VDIPEN in antigen-induced arthritis. Arthritis Rheum 1998, 41 : 647-656.
31. Van Meurs, JBJ, van Lent, PLEM, Holthuysen, AEM. Kinetics of aggrecanase and metalloproteinase induced neoepitopes in various stages of cartilage destruction in murine arthritis. Arthritis Rheum 1999, 42 : 1128-1139.
32. Kunicka, JE, Talle, MA, Denhardt, GH, et al. : Immunosuppression by glucocorticoids : inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. Cell Immunol 1993, 149 : 39-49.
33. Joyce, DA, Steer, JH, & Kloda, A : Dexamethasone antagonizes IL-4 and IL-10-induced release of IL-1RA by monocytes but augments IL-4, IL-10 and TGF-β induced suppression of TNF-α release. J Interferon Cytokine Res 1996, 16 : 511-517.