대한침구학회지 제19권 제5호(2002년 10월)
The Journal of Korean Acupuncture & Moxibustion Society

원 저

Protective and Anti-arthritic Effects of Cervi Pantotrichum Cornu Herbal Acupuncture, Inhibiting Dihydroorotate Dehydrogenase, on Phosphate Ions-mediated Chondrocyte Apoptosis and Rat Collagen-induced Arthritis

Choi Yoo-haeng, Choi Woo-shik, Song In-kwang,
Park Joon-sung, Lee Seung-deok and Kim Kap-sung

Department of Oriental Medicine
The Graduate School, Dong-Guk University

국문초록

녹용약침액이 생쥐의 Type II collagen 유발 관절염과 인산이온 유발 연골세포의 세포사에 있어 보호작용에 관한 연구

최유행·최우식·송인광·박준성·이승덕·김갑성

동국대학교 한의과대학 침구학교실

연구목적 : 면역억제와 활성 작용을 지닌 것으로 알려진 녹용약침(CPH)을 type II collagen 유발 관절염(CIA) 백서와 인산이온 유발 연골세포의 세포사에 있어 보호활성 효과를 연구하였다.

연구방법 : 7주된 암컷 Sprague-Dawley 쥐를 collagen으로 관절염을 유발시킨 후 CPH의 효과를 관절염 점수, 체중감소 등의 평가기준으로 검정하였다. CPH는 일주일에 5번씩 각각 10, 20, 30 및 100 μg/kg/day의 용량으로 양측 신수혈에 주입하였다.

· 접수 : 2002년 8월 24일 · 수정 : 9월 4일 · 채택 : 2002년 9월 14일
· 교신저자 : 김갑성, 경북 경주시 용강동 357, 동국대 경주한방병원 침구과
Tel. 054-770-1558 E-mail : kapsung@unitel.co.kr

연구결과 :

1. 300 mg/kg/day CPH처리로 관절염점수의 감소를 기초로 한 collagen 유발 관절염의 발생을 완전히 억제하였으며 관절염 점수상에서 CPH의 효과적인 용량은 64 mg/kg이었다.

2. CPH는 쥐의 간에 있는 DHO-DHase 활성을 Ki=843±43 μg/ml의 비교적 높은 비활성으로 억제하였다.

3. 관절염관련 세포의 증식억제활성을 검정한 결과 CPH의 항 증식효과는 세포주기 S기에서 정지시키는 활성을 나타내었다.

4. 쥐의 늑연골로부터 완전히 최종 분화된 비대연골세포를 분리 배양하여 3~5mM/L Pi를 첨가함으로서 세포사멸을 측정하였다. 10 μg/ml CPH 처리에 의한 보호(억제) 효과가 Pi-유발 연골세포의 세포사에 대한 Na-Pi cotransport의 경쟁적 저해제로 알려진 phosphonoformic acid(PFA)의 억제활성과 상응하는 수준으로 CPH의 활성을 확인하게 되었다.

Key words : **Type II collagen-induced arthritis; Apoptosis; Cervi Pantotrichum Cornu herbal acupunture (CPH); Phosphate ions(Pi); Cell cycle**

I. Abstract

The effect of water extract of Cervi Pantotrichum Cornu(CPC) prepared from the pilose antler of *Cervus korean* TEMMINCK var. mantchuricus Swinhoe (Nokyong), a traditional immunosuppressive and immunoactivating Korean herbal acupuncture, on Pi-induced apoptosis in chondrocytes from rat was studied.

Phosphate ions(Pi) are transported into chondrocytes via the Na-Pi transporter channel, then the chondrocytes are subjected to apoptosis by transported phosphate ions. Therefore, it is needed to block the Na-Pi transporter to bone resorption inhibition. It was known that Pi induced apoptosis of terminally differentiated hypertrophic chondrocytes. Pi induces cell death via the plasma membrane Na-Pi cotransport. This indicates that Pi-induced apoptosis can be prevented by inhibiting the plasma membrane Pi transport of chondrocytes.

Terminally differentiated hypertrophic chondrocytes were isolated from rat costochondrial cartilage and cell death was measured in the presence of 3-5 mM/L Pi. The effect of 10 μg/ml Cervi Pantotrichum Cornu herbal acupuncture(CPH) was compared to that of phosphonoformic acid (PFA), a competitive inhibitor of the Na-Pi co-transport on Pi-induced apoptosis in chondrocytes. 1 mM/L PFA blocked anion-induced cell death and prevented an increase in the cell Pi content. In a parallel study, we determined that the CPH also protected chondrocytes from death.

On the other hand, the effect of CPH was also evaluated as an inhibitor of dihydroorotate dehydrogenase (DHO-DHase) and tested in the rat collagen-induced arthritis(CIA) model. Female 7-week-old Sprague-Dawley rats were used for the evaluation of

CPH in the CIA model. Arthritis was evaluated by arthritis score, body weight loss, bone destruction score. CPH was administered by bilateral Shinsu(B23) acupuncture 5 times per week (10, 20, 30, and 100 μg/kg/day). CPH inhibited rat liver dihydroorotate dehydrogenase (DHO-DHase) in vitro with Ki=843±43 μg/ml. The anti-proliferative effect of CPH was caused by cell cycle arrest at the S-phase. Treatment with 300 mg/kg/day of CPH completely prevented the development of CIA based on the reduction of the arthritis score. The 50% effective dose (ED50) of CPH on arthritis score was 64mg/kg. CPH ameliorated body weight loss associated with disease onset. Importantly, CPH suppressed the development of arthritis, even when it was administered after a booster immunization of collagen. CPH is a novel immunosuppressant which inhibits DHO-DHase and its effects in CIA suggest that it could be useful in the treatment of rheumatoid arthritis.

Ⅱ. Introduction

Cervi Pantotrichum Cornu(CPC) prepared from the pilose antler of *Cervus korean* TEMMINCK var. *mantchuricus Swinhoe* (Nokyong), is a traditional Korean animal grown in south Korea and has long been recognized as one of the most effective and powerful invigorants, as well as an enhancer of systemic resistance to infectious diseases. Extract from CPC by water boiling methods, has been widely used in the treatment of some immune-related diseases, especially RA and satisfactory results are obtained[1,2]. However, little is known about the mode of action of this medication on RA.

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[3,4], and that altered activity of these enzymes is responsible for cartilage destruction and bone erosion associated with degenerative disorders such as RA. In clinical practice, RA are the most commonly encountered of the many forms of degenerative joint disease, with the former characterised by localized degenerative change 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.

Programmed cell death, or apoptosis, is an evolutionarily conserved physiological strategem from the organism. During development, the apoptotic program functions selectively to

remove unwanted cells, thereby promoting establishment of the mature phenotype[5]. In terms of understanding normal endochondral growth, terminally differentiated chondrocytes undergo programmed cell death[6]. Accordingly, apoptosis provides for the rapid and controlled removal of terminally differentiated cells from cartilage.

During endochondral bone formation, chondrocyte terminal differentiation is accompanied by an accumulation of calcium and phosphate ions (Pi); it was shown that, prior to deposition of apatite in the extracellular matrix, there was a marked accumulation of Pi[7]. To explore the mechanism by which Pi induces apoptosis, Mansfield et al.[8] reported the observation that Na-Pi transporters are expressed in chondrocytes. It was also concluded that, at the mineralization front, cell death is linked directly to the elevation in environmental anion concentration and to the concomitant rise in intracellular Pi levels.

Rats develop polyarthritis upon immunization with type II collagen. Pathological changes in the arthritic joints have a similarity to those of RA, characterized by a chronic inflammatory reaction in synovial tissue, producing pain, disability and eventual destruction of joints[9]. Thus, CIA is considered as an experimental model for human RA, and is utilized for development as anti-inflammatory and/or anti-arthritis drugs.

CPH inhibits the enzymatic activity of dihydroorotate dehydrogenase (DHO-DHase [EC 1.3.99.11]), the fourth enzyme of de novo pyrimidine synthesis. Inhibition of this enzyme prevents the production of pyrimidine nucleotides necessary for the synthesis of RNA and DNA, thus resulting in reduced cell proliferation[10]. Several inhibitors such as brequinar sodium and leflunomide were reported[11,12].

In the present study, the effect of CPH was compared to that of PFA, a competitive inhibitor of Na-Pi cotransport on Pi-induced apoptosis in chondrocytes. The effect of water extract of CPC was examined on chondrocytes. We determined that the CPH also protected chondrocytes from death. We have also compared the levels of apoptosis of the CPH- and Pi-treated cells from rat. CPH treatment at dose of 20 μg/kg suppressed the apoptosis. It was concluded that CPH is likely to be of potential importance in the pathogenesis of RA, and the development of novel therapeutic strategies for the latter disorder should include apoptosis inhibitory elements. We also clarified the inhibitory activity of CPH against DHO-DHase and examined both the prophylactic and sub-therapeutic effects of CPH in rat CIA.

Ⅲ. Materials and Methods

1. Materials

Female 6-week-old Sprague-Dawley rats were purchased from KCTC, KRIBB (Taejon, Korea). They were allowed at least 1 week

to adapt to the environment (25±3℃, 55±5% humidity and a 12 h light/dark cycle) and were used at 7 weeks of age. Food and water were provided *ad libitum*.

CPH tablets, a water extract of CPH were purchased from Gyeongju Oriental Medical Hospital, Dongguk University (Gyeongju city, Gyeongbuk), Korea as an i.p injection grade for human. Each tablet contained 100 μg of the extract. For i.p. injection into rats, randomly selected tablets were ground and suspended in normal saline at a concentration of 50 μg/10 μl[13]. CPH were administrated by bilateral Shinsu(B23) acupuncture to rats at dosages of 10, 20, 30 and 100 μg/kg rat, being the day before the initiation of the arthritis for 8 days to 2 weeks.

2. Na-Pi transporter with PFA and pharmacological CPH

Terminally differentiated hypertrophic chondrocytes isolated from rat costochondrial cartilage have been shown to undergo apoptosis when treated with 3~5 mmol/L Pi. To confirm whether Pi induces apoptosis or not, we blocked the activity of the Na-Pi transporter with PFA and measured chondrocyte death. Because this agent inhibited apoptosis, we then treated cells with Pi and determined if death was linked to an increase in the cell's Pi content. Finally, we determined whether the pharmacological CPH could protect chondrocytes from apoptosis.

3. Reagents

Methotrexate(MTX) was purchased from Sigma-Aldrich Co. (MO, U.S.A). Prednisolone and naproxen were purchased from Sigma-Aldrich Co. (MO. USA). Staurosporine was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan).

4. Cell Culture

Chondrocytes from costochondrial cartilage of 125 g Sprague Dawley rats were isolated as previously reported[14]. These cells were grown in a primary culture for 5 days in Dulbecco's modified high-glucose Eagle medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) containing 10% defined fetal calf serum (Jeil Biotech Inc., Taegu, Korea), 2 mM/L -glutamine, and 50 U/mL each of penicillin/streptomycin. Nonadherent chondrocytes were washed gently from the plate and used for Pi uptake assays, or replated at a density of 25,000 cells/㎠. To facilitate cell attachment, cultures were treated with 4 U of testicular hyaluronidase per milliliter of medium. After 3 days, cells were exposed to the agents in a serum-free medium. Expression of the hypertrophic phenotype was evaluated by optical microscopy, measurements of alkaline phosphatase activity, and expressions of type X collagen as described previously[15].

5. Treatment Protocol

For the Pi control group, the actual concentration of Pi in the serum-free medium was 1 mM and this value was set as the control. When the medium was supplemented with Pi, NaH_2PO_4 was added to the bulk me-

dium and rapidly mixed, we prevented mineral precipitation. Special care was taken to maintain the medium pH; once the Pi was dissolved, the pH of the medium was then adjusted to pH 7.4 with KOH. No significant change in pH was observed during the experiment. PFA was used at a concentration of 10 μM to 1 mM in the presence of 1mM to 5 mM Pi. Other all reagents were purchased from Sigma-Aldrich Co. (MO, USA).

6. Measurement of Apoptosis

(a) MTT Assay

Chondrocyte viability was determined by the MTT assay. This assay is widely used to measure cell death. Chondrocyte apoptosis could be monitored using the procedures[16]. The assay is based on the ability of mitochondrial dehydrogenases to oxidize thiazoyl blue (MTT), a tetrazolium salt ((3-[4,5-Dimethyl-2-thiazolyl]-2,5-dphenyl-2Htetrazolium bromide), to an insoluble blue formazan product. Briefly, cells were grown in 24 well plates in medium with or without the test agent. At the end of the treatment period, they were treated with MTT (120 g/mL) in a serum-free medium and incubated at 37℃. After 3hrs, the supernatant was removed and the formazan crystals were solubilized in 0.04 M/L HCl in isopropanol and stirred for 10 min at room temperature. The optical density was read at 595 nm using an enzyme-linked immunoassay (ELISA) plate reader and the results were normalized to values generated by untreated control cells[17].

(b) TUNEL Assay

Cartilage chondrocytes(0.35×10^6cells) were plated in 6 well plates and treated with Pi in the presence or absence of PFA or CPH for 24hrs. The cells were fixed and stained using the Klenow FragEL DNA Fragmentation Detection Kit(Oncogene Research Products, Cambridge, MA). While the manufacturer's instructions were followed, significant changes were made to the protocol. Thus, cells were fixed in 4% formaldehyde in phosphate-buffered saline(PBS), pH 7.2, for at least 30 min. After rinsing with tris-buffered saline (TBS), cells were permeabilized by incubation with Proteinase K for 2 min. Cells were then washed with TBS, incubated with the equilibration buffer for 15 min, and then treated with the Klenow enzyme in equilibration buffer for 3 h at 37℃ in a humidified chamber. The reaction was stopped and blocked for 20min, and then incubated with the conjugate in blocking buffer for 30min at room temperature. After rinsing, cells were treated with CPH for 30min. Stained sections were viewed under microscope(Olympus Co., Tokyo, Japan) and images were digitally captured[8].

7. Chondrocyte Pi Uptake

Because measurement of Pi in adherent cells generates aberrant results due to Pi binding to matrix protein and mineral, all assays were performed using suspension cultures in which there was minimum matrix. For this study, cells were lifted from the

plates and resuspended in the medium at a density of 200,000 cells/mL in the presence or absence of added Pi. After 24hrs, cells were placed on ice and washed three times with a buffer containing 100 mM/L NaCl, 50 mM/L mannitol, 5 mM/L HEPES, and 1 mM/L PFA (pH 7.5) or 2μg/ml CPH. Cells were then extracted with ice-cold 3% perchloric acid centrifuged at 15,000 g for 15 min, and the Pi concentration of the supernatant was determined using the method of Van Veldhoven and Mannaerts[18]. The Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA) was used to determine the amount of protein in each sample.

8. Collagen Immunization

Bovine type II collagen solution, which was purchased from Sigma (St. Louis, Mo, USA) was diluted (3 mg/ml to 1.6 mg/ml) with 10 mM acetic acid and then emulsified in an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). The emulsion (500 μl containing 400 μg collagen) was injected intradermally on the back of rats. Seven days later, 100 μl of the same emulsion was injected intradermally at the base of the tail.

9. CPH Extract Administration

CPH were administrated on bilateral Shinsu(B23) daily to rats at dosages of 10, 20, 50 and 100μg/kg rat being the day before the initiation of the arthritis for 8 days to 2 weeks or administered 5 times per week on the day of the primary immunization with type II collagen (i.e. from day 0) to the day before the last day.

Immunization with type II collagen was carried out on Monday in each experiment, and the drug was administered from Monday to Friday throughout the experiment. Each experiment finished on day 18th or day 23th. Drugs administrated by bilateral Shinsu(B23) acupuncture to rats were as follows : CPH (10, 20, 30 and 100 μg/kg), MTX(0.1 mg/kg), naproxen (3 mg/kg) and prednisolone (3 mg/kg)

The administered dose of MTX (0.1 mg/kg) was the maximum dose that did not induce myelosuppression[19]. Saline water was administered as the vehicle control. In the subtherapeutic experiment, CPH were administered by bilateral Shinsu(B23) acupuncture 5 times per week from day 7 to the day before the last day of each experiment.

10. Arthritis Evaluation

Rats were examined for visual appearance of arthritis and the lesion of each hind paw was graded from 0 to 4 according to the increasing extent of erythema and edema of the periarticular tissue, as described below.

The arthritis score of each rat was the sum of the scores of each of the 4 limbs, the

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

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

11. DHO-DHase Activity Measurement

A partially purified preparation of mitochondria was produced from rat liver(7 week male SD rat)[20].

Measurement of DHO-D Hase activity was performed according to Peters et al.[20] with slight modifications.

The kinetic parameters (apparent Km, Vmax, apparent Ki and Ki') of mitocondroal DHO-DHase were measured using L-dihhydroorotate (L-DHO; 50 μM) as a substrate. The rate of conversion of L-DHO to L-orotate was measured by HPLC equipped with SLC-10A system controller and UV detector (Shimadzu, Kyoto, Japan). The separation was achieved by isocratic elution with 10 mM potassium phosphate buffer(pH.7.2) on a Partisil-10 SAX column(4.6×250mm, Whatman, USA). The UV absorbance of the effluent was monitored at 280 nm.

12. Proliferation Assay and Cell Cycle Analysis

Jurkat cells, a human T-cell line from an acute leukemia, were obtained from ATCC(T IB 152) and cultured in RPMI 1640 medium (Sigma-Aldrich Co., MO, USA) with 10% fetal bovine serum, Proliferation was assessed by the amount of [^{3}H] thymidine(25 Ci/mmol; Amersham Pharmacia Biotecs, Buckinghamshire, UK) incorporation into cells. Jurkat cells(5×104 cells/200 ul) were cultured for in the presence or absence of CPH. After 64hrs [^{3}H] thymidine was added to the cells and the cells were incubated for a subsequent 8hrs. Cell cycle analyses were carried out using Cycle TEST^{TM} PLUS DNA Reagent kit from Becton Dickinson Immunocytometry Systems(San Jose, CA, USA). Also cells were analyzed by flow cytometry on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) in the presence or absence of CPH.

13. Protein Determination

Synovial fluid protein levels, for calculation of specific proteolytic enzyme activity measurements and specific protein carbonyl measurements above, were determined by the method of Lowry et al.[21] using bovine serum albumin as the standard.

14. Statistical Analysis

All experiments were repeated three to five times and the mean and S.E. were determined. MTT absorbance values for control group were normalized to 100% ; experimental group values were expressed as percent of control group. Significant differences between sets of values for control group and experimental group were assessed by one-way analysis of variance (ANOVA) by Dunnett's test or Kruskal-Wallis. When required, we took the square root to normalize the data[22]. The p value refers to a com-

parison of a measured parameter in the experimental group with that of the appropriate control group ; significance was set at $p < 0.05$.

Ⅳ. Results

1. Effect of Pi and PFA and CPH on chondrocyte apoptosis.

Inorganic Pi (3-5 mM/L) caused a sharp decrease in the percentage of viable cells. Thus at 5 mM/L, almost 85% of the cells were killed by Pi. Treatment with the transport blocker, PFA (1 mM/L), protected the cells from Pi (Fig. 1A). Fig. 1A shows that, when the Pi concentration was 3 mM/L, almost all of the cells were dead; in the presence of PFA, 100% of cells were viable. A same result was seen when the cells were treated with 5 mM/L Pi. However, treatment with 10 μg/ml CPH protected the cells from Pi (Fig. 1B).

Chondrocytes were treated with Pi in the presence and absence of 1 mM/L PFA or 10 μg/ml CPH. After 24hrs, the viability of the chondrocytes was determined using the MTT assay. Although 3 and 5 mM/L Pi caused a dramatic fall in cell viability, the presence of PFA completely blocked the apoptotic effects of the anion. CPH protected the apoptotic effects of the anion. Values shown represent mean ± S.E. of five separate experiments.

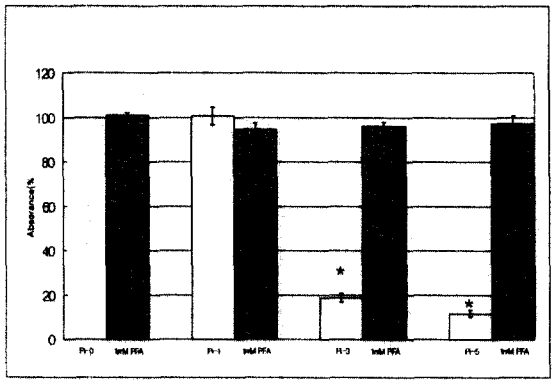


Fig 1A. Effect of PFA on chondrocyte apoptosis.

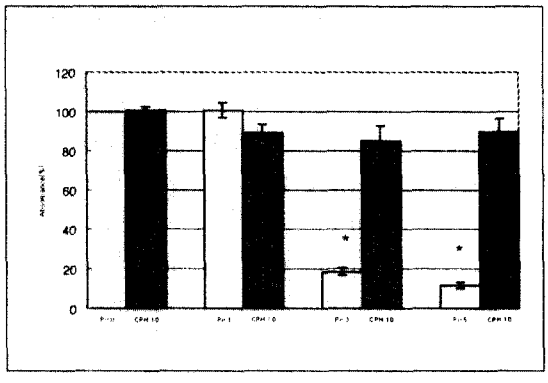


Fig 1B. Effect of CPH on chondrocyte apoptosis.

* Significantly different from 1mM/L control group ($p < 0.05$).

2. Dose-dependent effect of CPH on Pi-induced chondrocyte apoptosis.

To determine whether CPH protect the cells from 3 mM/L Pi or not, the chondrocytes were treated with increasing concentrations (5~50 μg/ml) of CPH as the transporter blocker. Fig. 2 shows that CPH caused a dose-dependent increase in the

percentage of viable cells. Effective protection against 3 mM/L Pi was achieved when the CPH concentration was 20 μg/ml. An CPH concentration as low as 5 μg/mL meaningly inhibited Pi-mediated chondrocyte apoptosis.

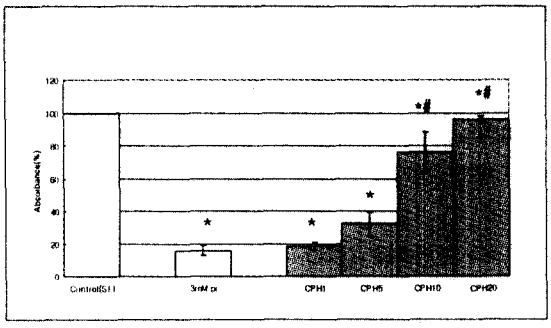


Fig 2. Dose-dependent effect of CPH on Pi-induced chondrocyte apoptosis.

Tibial chondrocytes were treated with increasing concentrations of CPH in the presence of 3 mM/L Pi in serum-free (SF) medium. After 24hrs, cells were isolated and cell death was estimated by the MTT assay. Although 3 mM/L Pi induced death of almost 90% of the cells, the addition of >20 μg/ml CPH significantly reduced cell death. Values shown represent mean ± S.E. of five separate experiments.

* significantly different from serum-free control ($p < 0.05$)

significantly different from 3 mM/L Pi ($p < 0.05$).

3. Effect of Pi and CPH on chondrocyte survival as observed by the TUNEL method

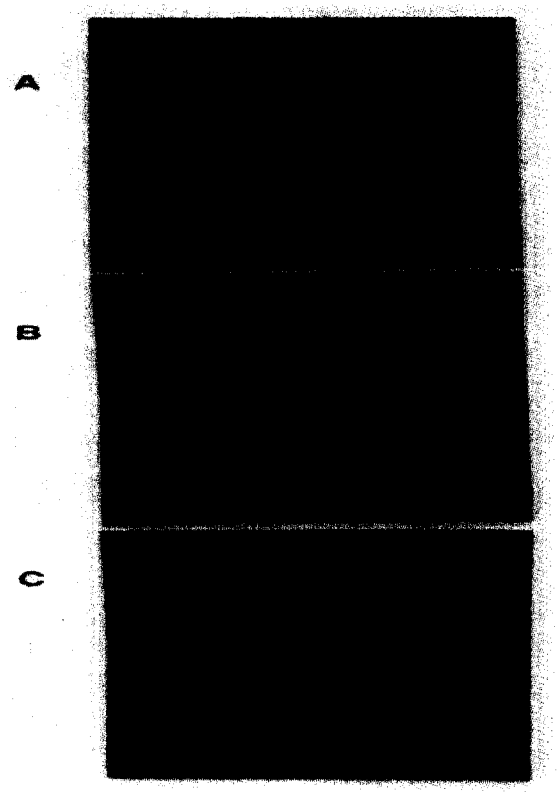


Fig 3. Chondrocytes stained by the TUNEL method following treatment with Pi and CPH.

Cells were protected from apoptosis is shown in Fig. 3. Cells were treated with 3 mM/L Pi in the presence or absence of CPH and apoptosis as determined using the TUNEL assay. As might be expected, when the Pi concentration was low (control), few TUNEL-positive cells were evident; in contrast, when treated with 3 mM/L Pi, almost all of the cells were TUNEL positive, indicating that these chondrocytes were apoptotic. In the presence of CPH, 3 mM/L Pi induced few TUNEL-positive cells in the culture. These results indicate that CPH pro-

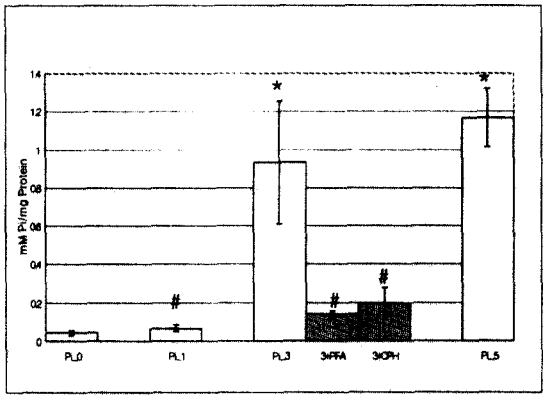
tected the chondrocytes from Pi-induced apoptosis

Chondrocytes were treated with 3 mmol/L Pi in the presence or absence of 10 μg/ml CPH. The cells were then fixed and stained by the TUNEL procedure. (A) Control cells maintained in serum-free DMEM. (B) Chondrocytes treated with 3 mmol/L Pi for 24hrs. (C) Chondrocytes treated with 3 mM/L Pi in the presence of 10 μg/ml CPH for24hrs. In the presence of 3 mM/L Pi, many of the cells were TUNEL-positive (B). However, CPH caused a marked reduction in the number of positive(apoptotic) cells(C). Original magnification×400.

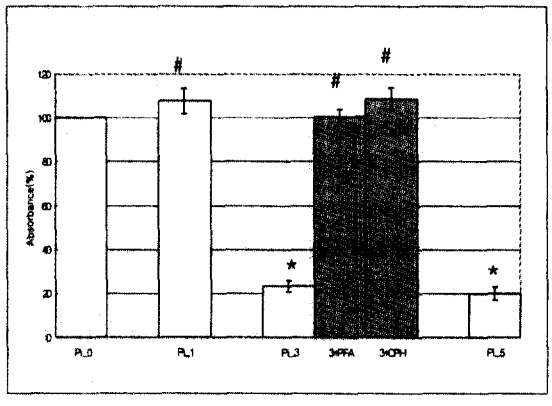
4. Effects on Pi accumulation and viability of cells treated with Pi in the presence or absence of PFA and CPH.

To ascertain if treatment with Pi influenced the intracellular Pi load, chondrocytes were treated with Pi in the presence and absence of CPH. Fig. 4A shows that, in the presence of 3 mM/L Pi, there was a 27-fold increase in cellular Pi content (0.028 μM/μg protein V 0.89 μM/μg protein). In addition, as might be expected, treatment with PFA as a positive control or CPH significantly decreased cell Pi loading. Because the Pi-loading experiment was performed using suspension cultures, we also assessed the impact of Pi treatment on cell viability. Fig. 4B shows that 3 and 5 mmol/L Pi induced death in about 75% of treated cells, whereas PFA and CPH protected the chondrocytes from Pi-dependent apoptosis.

Suspended cultures of tibial chondrocytes were treated with Pi for 24hrs. Cells were also treated with 5 mmol/L Pi in the presence of 3mM/L PFA(3+PFA) or 10μg/ml CPH(3+CPH). The Pi content(A) and the



A. Effect of the Pi content of the treated cells



B. Effect on the percent viability of the treated cells

Fig 4. Pi accumulation and viability of cells treated with Pi in the presence or absence of PFA and CPH

percent viability (B) of the treated cells was then determined. Although Pi caused a 20-30-fold increase in cell Pi content, PFA and CPH markedly reduced the total concentration of Pi accumulated by the treated cells and protected the cells from apoptosis. Values shown represent mean ± S.E. of five separate experiments.

* significantly different from 1 mM/L(control group) Pi ($p<0.05$);

significantly different from 3 mM/L Pi ($p<0.05$).

5. Effects of CPH on rat CIA model

Treatment with CPH (100 μg/kg) almost effectively prevented the development of CIA Table I. MTX (0.1 mg/kg) partially prevented the development of CIA. Although the body weight of vehicle treated rats (control) decreased with the development of arthritis, the body weight of rats treated with CPH was maintained as same level as normal rats Table II. CPH, MTX, naproxen and prednisolone, suppressed the destruction of bone Table II.

6. Effect of CPH on the DHO-DHase Activity

Table I. Effects of CPH and Various Drugs on Incidence of Rat CIA.

	Treatment Dose (μg or mg/kg)	Days after immunization					
		8	10	12	14	16	20
Control	-	0/6[a]	3/6	6/6	6/6	6/6	6/6
CPH	100 μg/kg	0/6	0/6	1/6	1/6	2/6	2/6
MTX	0.1 mg/kg	0/6	2/6	3/6	4/6	4/6	4/6
Naproxen	3 mg/kg	0/6	2/6	3/6	4/6	2/6	2/6
Prednisolone	3 mg/kg	0/6	0/6	4/6	4/6	3/6	4/6

[a] The incidence of arthritis was examined on the days indicated

Table II. Effects of CPH and various drugs on body weight change and bone destruction rat CIA[a].

Treatment	Dose(mg/kg) (Only CPH μg/kg)	Body weight change(g)[b]	Bone destruction score[c]
Normal	-	36.5±4.6**	-
Control	-	14.6±3.6	12.5±0.5
CPH	100	37.3±2.1**	0.3±0.1*
MTX	0.1	20.4±3.7	6.8±1.3*#
Naproxen	3	34.7±3.1*	3.1±1.2*
Prednisolone	3	21.7±3.7	4.7±1.6*

[a] Results are expressed as the mean ± S.E. (N=6)

[b] Body weight change from day 7 to day 18 was measured.

[c] Radiographs were taken on day 20th.

* $P<0.05$, ** $P<0.01$ compared with control group,

# $P<0.05$ compared with CPH treated group.

The activity of DHO-DHase partially purified from rat liver was evaluated; the apparent Km value of the substrate (L-DHO) was 2,860±9.4 μg/ml (mean ± S.E. in five experiments) and the Vmax was 26,430 μg/h/mg protein, determined from a Lineweaver-Burk plot. CPH effectively inhibited DHO-DHase activity in a concentration dependent manner. CPH exhibited mixed competitive and noncompetitive inhibition. the apparent Ki value of CPH was 843±43 μg/ml Table Ⅲ.

Table Ⅲ. Kinetics Parameters of CPH on DHO-DHase Activity in Rat Liver Mitochodria[a].

	Apparent Ki (μg/ml)
CPH	843±43

[a] Results are expressed as the mean ± S.E.

7. Effect of CPH on Cell Progression in Jurkat Cells

CPH inhibited the proliferation of Jurkat cells, a human T-cell line, with an IC_{50} value of 170 μg/ml. Jurkat cells treated with CPH for 6hrs induced cell cycle arrest at the S-phase and entry into the G_2- and M-phase was inhibited Fig. 5; the minimum effective concentration of CPH was 3.0 μg/ml. These results suggest that the antiproliferative effect of CPH was due to cell cycle arrest.

Jurkat cells were treated with CPH for 6 h and then analyzed on a flow cytometer.

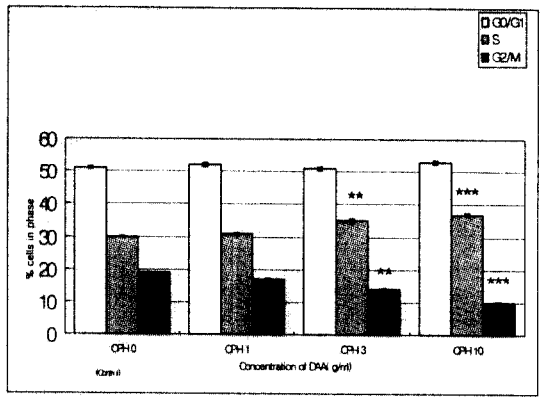


Fig 5. Effect of CPH on cell-cycle progression in Jurkat cells

Results are shown as the mean±S.E.(N=4).

**, P<0.01, ***, P<0.001 compared with vehicle treated group.

V. Discussion

CPH is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanisms by which the CPH modifies the clinical status of RA are not well understood.

The bioactivity of the deer antler may act as an immune enhancer[23,24]. Previously, antifungal activity, and the suppressing activity of the hyphal transition from yeast in C. albicans, were found from deer antler extract [25].

By using biochemical and chemical methods, we identified the compound from deer antler extract that is responsible for the suppression of hyphal transition as 2-lyso-

phosphatidylcholine, and this suppression may be mediated through the MAP kinase pathway[26].

The mechanisms by which the CPH modifies the clinical status of RA are not well understood. Previously, our CPH inhibited production of IL-1β and TNF-a from macrophages in response to in vivo stimulation with bacterial lipopolysaccharides when the extract was administered into mice once a day for 7 days[13], suggesting that the CPH administered orally into the patients inhibits cytokine production from both T cells and macrophages and potent effects on RA. The degenerative joint disorder RA is characterised by cartilage destruction and bone erosion, resulting from mechanical wear or following joint tissue inflammation, respectively. The precise mechanisms responsible for the differential pathogenesis of RA remain unknown.

CPH might be a useful tool for the treatment of RA. It would be incredible if drugs as powerful as this did not have serious toxicity, but further studies will be necessary to answer this question. The recommended dose of CPH in the management and treatment of rat RA will be 100μg/kg, which is two-fifth of the human therapeutic dose. However, biochemical and metabolic analysis of the constituents of CPH have to be analyzed to further delineate its mechanisms of action in arthritis.

The exposure to Pi increased the intracellular Pi level and caused chondrocyte apoptosis. Although it is not clear whether Pi-mediated apoptosis was receptor-mediated, use of the Na-Pi cotransport inhibitor, PFA, and the bisphosphonate, alendronate, provided interesting insights into the mechanisms of Pi action[27]. Because CPH blocked apoptosis, the possibility exists that this medicine can be used to protect chodrocytes from completing their life cycle. At high pharmacological concentrations, the drug directly activated components of the apoptotic system (caspases). Bravo et al[3] reported that, when isolated mitochondria were treated with elevated levels of Pi, there were irreversible changes in mitochondrial oxidative-phosphorylating activity.

A number of more recent studies have linked apoptosis directly with skeletal cell functions. Thus, the drug to activate osteoclast apoptosis would be ideal for influencing the remodeling rate, as suggested[28]. Thus, it may be possible to regulate abnormal or premature terminal differentiation of cells in the growth plate, as well as to control apoptosis in diseased cartilage.

Administration of CPH at 10μg/kg before immunization of type II collagen effectively prevented the development of CIA in rats. It was discovered that CPH inhibited dihydroorotate dehydrogenase (DHO-DHase). The apparent Ki value of CPH=843$\pm$43μg/ml. As an example of DHO-DHase inhibitor, leflunomide is an effective and safe treatment for patients with active RA[29]. The anti-inflammatory and anti-immunosuppre-

ssive properties of leflunomide are reported to derive from the inhibition of DHO-DHase[30] and tyrosine kinases[31]. Inhibition of DHO-DHase depletes the orotate and subsequently decreases uridine monophosphate[31]. As a critical level of uridine monophosphate(ribonucleotides) is required to stabilize p53[32], a lower level of uridine monophosphate activates p53 and subsequently cells are arrested in the G_1-phase of the cell cycle[29,33]. The effects of CPH on the cell cycle were similar to that of an active leflunomide. These suggest that CPH may also activate p53. In conclusion, CPH is a potent inhibitor of DHO-DHase and it effectively prevents the development of CIA.

VI. Conclusion

Protective and Anti-arthritic Effects of Cervi Pantotrichum Cornu Herbal Acupuncture, Inhibiting Dihydroorotate De-hydrogenase, on Phosphate Ions-mediated Chondrocyte Apoptosis and Rat Collagen-induced Arthritis was studied. The results were as follows.

1. The effect of 10 μg/ml Cervi Pantotrichum Cornu herbal acupuncture(CPH) was compared to that of phosphonoformic acid (PFA), a competitive inhibitor of the Na-Pi co-transport on Pi-induced apoptosis in chondrocytes.

2. 1 mM/L PFA blocked anion-induced cell death and prevented an increase in the cell Pi content. In a parallel study, we determined that the CPH also protected chondrocytes from death.

3. CPH inhibited rat liver dihydroorotate dehydrogenase (DHO-DHase) in vitro with $K_i = 843 \pm 43$ μg/ml and the anti-proliferative effect of CPH was caused by cell cycle arrest at the S-phase.

4. Treatment with 300 mg/kg/day of CPH completely prevented the development of CIA based on the reduction of the arthritis score. The 50% effective dose (ED_{50}) of CPH on arthritis score was 64 mg/kg.

5. CPH ameliorated body weight loss associated with disease onset. Importantly, and suppressed the development of arthritis, even when it was administered after a booster immunization of collagen.

VII. References

1. Wang B-X, Zhao X-H., Yang X-W, Kaneko S., Hattori M., Namba T. and NomuraY. Inhibition of lipid peroxidation by deer antler (Rokujo) extract

in vivo and in vitro. Journal of Medical and Pharmaceutical Society for WA KAN-YAKU. 1988 ; 5 : 123-128.

2. 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. 1988 ; 36 : 2587-2592.
3. V. Testa, F. Capasso, N. Muffulli, A. Sgambato and P.R.J. Ames, Proteases and antiproteases in cartilage homeostasis. Clin Orthop Relat Res. 1994 ; 308 : 79-84.
4. T.A. Einhorn and R.J. Majeska, Neutral proteases in regenerating bone. Clin Orthop. 1991; 262: 286-297.
5. G. Kroemer, P. Petit, N. Zamzami, J.L. Vayssiere and B. Mignotte, The biochemistry of programmed cell death. FASEB J. 1995 ; 9 : 1277-1287.
6. G. Gibson, D.L. Lin and M. Roque, Apoptosis of terminally differentiated chondrocytes in culture. Exp Cell Res. 1997 ; 233 : 372-382.
7. I.M. Shapiro and A. Boyde, Microdissection : Elemental analysis of the mineralizing growth cartilage of the normal and rachitic chick. Metabol Bone Dis Rel Res. 1984 ; 5 : 317-326.
8. Masfield, K., C.C. Teixeria, C.S. Adams and I.M. Shapiro. Phosphate ions mediate chondrocyte apoptosis through a plasma membrane transporter mechanism. Bone. 2001 ; 28 : 1-8.
9. Trentham DE. Collagen arthritis as a relevant model for rheumatoid arthritis. Arthritis Rheum. 1982 ; 25 : 911-916.
10. Jaffee BD, Jones EA, Loveless SE, Chen SF. The unique immunosuppressive activity of brequinar sodium. Transplant Proc. 1993 ; 25 : 19-22.
11. Xu, X., Williams, JW., Shen, J., Gong H., Yin D.P., Blinder L. In vitro and in vivo mechanism of action of the antiproliferative and immunosuppressive agent, brequinar sodium. J. Immunol. 1998 ; 160 : 846-853.
12. Cherwinski HM, Cohen RG, Cheung P, Webster DJ, Xu YZ, Caufield JP. The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimide biosynthesis. J. Pharmacol. Exp. Ther. 1995 ; 275 : 1043-1049.
13. Jin-Gyu Kim. Inhibitory effects of Cervi Pantotrichum Cornu herbal acupuncture on type II collagen-induced arthritis. Dongguk University. 2000 ; 21.
14. Boyan, BD, Schwartz Z, Swain LD, Carnes DL Jr and Zislis T. Differential expression of phenotype by resting zone and growth region costochondral chondrocytes in vitro. Bone. 1988 ; 9 : 185-194.
15. O. Oshima, P.S. Leboy, S.A. McDonald, R.S. Tuan and I.M. Shapiro, Develop-

mental expression of genes in chick growth cartilage detected by in situ hybridization. Calcif Tissue Int. 1989 ; 45 : 182-192.

16. T. Mosmann, Rapid colorimetric assay for cellular growth and survival : Application to proliferation and cytotoxicity assays. J Immunol Meth. 1983 ; 65 : 55-63.
17. A. J. McGahon, S. J. Martin, R. P. Bissonnette, A. Mahboubi, Y. Shi, R. J. Mogil, W.K. Nishioka and D.R. Green, The end of the (cell) line: Methods for the study of apoptosis in vitro. Meth Cell Biol. 1995 ; 46 : 153-185.
18. P. P. Van Veldhoven and G. P. Mannaerts, Inorganic and organic phosphate measurements in the nanomolar range. Anal Biochem. 1987 ; 161 : 45-48.
19. Hom JT, Gliszczynski VL, Cole HW, Bendele AM. Interleukin 1-mediated acceleration of type II collagen-induced arthritis : effects of anti-inflammatory or anti-arthritic drugs. Agents Actions. 1991 ; 33 : 300-309.
20. Peters GJ, Laurensse E., Leyva A, Ponedo HM. A sensitive, nonradiometric assay for dihydroorotic acid dehydrogenase using anion-exchange high-performance liquid chromatography. Anal. Biochem. 1987 ; 161 : 32-38.
21. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.I. Randall, Protein measurement with Folin phenol reagent. J Biol Chem. 1951 ; 193 : 265-275.
22. G.W. Snedecor and W.G. Cochran. Statistical Methods, 6th Ed, Iowa State University, Ames, IA 1967.
23. J.S. Suh, J.S. Eun, J.N. So, J.T. Seo and G.J. Jhon. Phagocytic activity of ethyl alcohol fraction of deer antler in murine peritoneal macrophage Biol. Pharm. Bull. 1999 ; 22 : 932-935.
24. S.-Y. Han, S.-H. Cho, S.-Y. Kim, J.-T. Seo, S.-J. Moon and G.-J. Jhon. Monoacetyldiglycerides as New Ca^{2+} Mobilizing Agents in Rat Pancreatic Acinar Cells. Bioorg. Med. Chem. Lett. 1999 ; 9 : 59-64.
25. H.S. Park, G.J. Jhon and W.J. Choi. Deer antler extract selectively suppresses hyphal growth in dimorphic fungus, Candida albicans. J. Microbiol. Biotechnol. 1998 ; 8 : 291-294.
26. Min J, Lee YJ, Kim YA, Park HS, Han SY, Jhon GJ, Choi W. Lysophosphatidylcholine derived from deer antler extract suppresses hyphal transition in Candida albicans through MAP kinase pathway. Biochim Biophys Acta. 2001 ; 1531 : 77-89.
27. L.I. Plotkin, R.S. Weinstein, A.M. Parfitt, P.K. Roberson, S.C. Manolagas and T. Bellido, Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest. 1999 ; 104 : 1363-1374.
28. E.M. Greenfield, Y. Bi and A. Mi-

yauchi, Regulation of osteoclast activity. Life Sci. 1999 ; 65 : 1087-1102.
29. Svhkolemmer HU, Milbert U, Zeitter D, Haun G, Wunschel M, Martlett RR. Cell cycle regulation and inhibition of de novo pyridine biosynthesis by leflunomide. Inflamm. Res. 1999 ; 48 : S5115-5116.
30. Davis JP, Cain GA, Pitts WJ, Magolda RL, Copeland RA. The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. Biochemistry. 1996 ; 35 : 1270-1273.
31. Xu X, Blinder L, Shen J, Gong H, Finnegan A, Williams JW. In vivo mechanism by which leflunomide controls lymphoproliferative and autoimmune disease in MRL/MpJ-lpr mice. J. Immunol. 1997 ; 159 : 167-174.
32. Wahl GM, Linke SP, Paulson TG, Huang LC. Maintaining genetic stability through TP53 mediated checkpoint control. Cancer Surv. 1997 ; 29 : 183-219.
33. Linke SP, Clarkin SP, Di Leonardo A, Tsou A, Wahl GM. A reversible, p53-dependent G0.G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes Dev. 1996 ; 10 : 934-947.