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Original Article

Prevention of Collagen-induced Arthritis in Mice by Deer Antler Extract(DAE)

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Abstract

Objectives : The effect of water extract of the pilose antler of Cervus korean TEMMINCK var. *mantchuricus Swinhoe* (Nokyong), a traditional immuno-suppressive and immuno-activating Korean oriental medicine, on collagen-induced arthritis (CIA) mice model was studied. Identification of common Nokyong capable of affording protection or modulating the onset and severity of arthritis may have important human health implications.

Methods : Nokyong has shown to possess anti-inflammatory and anticarcinogenic properties in experimental animals. In this study we determined the effect of DAE on collagen-induced arthritis in mice.

Results : In three independent experiments mice given DAE in water exhibited significantly reduced incidence of arthritis (33% to 50%) as compared with mice given no DAE in water (84% to 100%). The arthritis index also was significantly lower in DAE-fed animals. Western blot analysis showed a marked reduction in the expression of inflammatory mediators such as cyclooxygenase 2 (Cox-2), Interferon-γ (INF-γ), and tumor necrosis factor α (TNF-α) in arthritic joints of DAE-fed mice. The neutral endopeptidase (NEP) activity was approximately 6-fold higher in arthritic joints of non-DAE-fed mice in comparison to nonarthritic joints of nonimmunized mice whereas it was only 2-fold higher in the arthritic joints of DAE-fed mice. Additionally, total IgG and type II collagen-specific IgG levels were lower in the arthritic joints of DAE-fed mice.

Conclusion : Taken together our studies suggest that DAE may be useful in the prevention of onset and severity of arthritis.

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Key words : type II collagen-induced arthritis; Rheumatoid arthritis; deer antler extract; Pilose antler of *Cervus korean* TEMMINCK var. *mantchuricus Swinhoe* (Nokyong); tumor necrosis factor α; neutral endopeptidase; cyclooxygenase.

Ⅰ. Introduction

Unossified horn or pilose antler cut from deer which belong to the Cervidae is generally termed "Nokyong". Nokyong is one of the most famous Korean oriental medicines and has been considered to possess sexual-reinforcing and anti-aging actions. Thus, Nokyong has been used invigorate the kidney-yang (腎陽), replenish vital essence and blood and strengthen muscle and bones in Korean oriental medicine. Water extract of the pilose antler of *Cervus* korean TEMMINCK var. *mantchuricus Swinhoe* (Nokyong), a traditional immuno-suppressive and immuno-activating Korean oriental medicine, have sometimes been compounded in recent Korean commercial restoratives, although little is yet known about the pharmacological effects or active ingredients. Extract from Nokyong 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[1-2]. However, little is known about the mode of action of this traditional medication on RA.

Collagen-induced arthritis (CIA) in mice is a widely studied animal model of inflammatory polyarthritis with similarities to RA. CIA is induced after immunization of susceptible strains of mice with articular chicken type II collagen (CII) in complete Freund's adjuvant (CFA), and the resulting disease is primarily mediated by an autoimmune response[3-4]. The significance of the model lies in the fact that CII is the major constituent protein of the cartilage in the diarthrodial joints - the primary site affected in RA[4]. The pathogenic immune response to CII in CIA is rather complex and depends on specific MHC haplotypes (H-2q), CII-specific Th1-type IFN-γ-producing T cells and B cell responses (IgG2a producing), and several other cellular and biochemical factors[4-5]. Thus, there is a synergy in the CII-specific humoral and cellular immune response that is critical for the pathogenesis of the disease, and treatments designed to interfere with this synergistic response have been shown to prevent the onset of CIA[6-9]. Because of many compelling similarities between CIA and RA, CIA is an excellent model not only to precisely define the role of T and B cells in the pathogenesis of the disease but also to develop and test approaches for the prevention and treatment of arthritis in humans.

Identification of common substances capable of affording protection or modulating the onset and severity of arthritis may have important human health implications. Recently, some studies have reported the effects of the administration of synthetic and naturally occurring compounds on the progression of CIA in experimental animals. Inhibition of CIA has been reported in taxol-treated rats where it was shown that the synoviocyte and neovascular components reverted to naive synovium morphology[10]. Rolipram, which is a type IV phosphodiestrase inhibitor, has been shown to ameliorate CIA by suppressing the expression of tumor necrosis factor α (TNF-α) and Th1-type cellular immune responses in mice with CIA[11].

Extensive studies carried out in the past decade in many laboratories have shown that DAE possesses anti-inflammatory as well as anticarcinogenic properties[12]. One present study determined the effect of oral infusion of DAE on the incidence and severity of CIA in DBA/1 mice that are highly susceptible to the development of polyinflammatory arthritis after immunization with heterologous type

II collagen in CFA[3-4]. The results indicate that mice given DAE before treatment with the disease-inducing protocol were significantly less susceptible to the development of CIA, and if they developed arthritis, the disease was late in onset and mild in comparison to mice given no DAE.

II. Materials and methods

1. Mice

Male DBA/1 mice (H-2q), 6-8 weeks old, were purchased from Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Mice were maintained throughout the study in the Animal Care Facility, School of Oriental Medicine, Dongguk University, and were handled according to National Institutes of Health guidelines for humane treatment of experimental animals.

2. Deer antler extract(DAE)

Briefly, dried Nokyong was extracted twice with hot water and the combined extract was concentrated and the total soluble fraction was concentrated under vacuum, dissolved in water, and freeze-dried. This light brown, solid matter is called DAE. A solution of 0.2% DAE in the water was prepared and given to experimental mice ad libitum as the sole source of drinking water (DAE-fed group).

Nokyong tablets, a water extract of Nokyong were purchased from Gyeongju Oriental Medical Hospital, Dongguk University, Gyeongju city, Gyeongsangbuk-Do, Korea.

The mice were provided normal Phosphate Buffered Saline (PBS) as the non-DAE-fed group. This DAE feeding protocol has been used in mice in many prior studies from many laboratories[13].

3. CII and Immunization

CII used in these studies was purchased from Sigma(St. Louis, MO, USA). A working solution of 2 mg/ml of CII was prepared in 0.05 M acetic acid and stored on ice before use. This solution was emulsified with an equal volume of CFA (GIBCO-BRL), and mice were immunized intradermally in the base of the tail. Mice were boosted 3 weeks later ,with CII emulsified in incomplete Freund's adjuvant and observed for up to 85 days postimmunization for clinical symptoms of arthritis.

4. Measurement of Clinical Severity of Arthritis

The severity of arthritis in each affected paw was graded as grade 1, redness and swelling; grade 2, deformity; and grade 3, ankylosis in the affected joint[14]. Arthritis index was calculated by adding the total clinical severity score of each paw in each group of mice and dividing by the total number of arthritic mice in that group.

5. Preparation of Cell-Free Extract of the Knee Joints

Arthritic and nonarthritic joints were removed from the sacrificed animals, dissected free of soft tissue, and then frozen in liquid nitrogen. Before use the frozen joints were thawed and cut into small pieces and homogenized in 5 vol of 50 mM Tris · HCl buffer, pH 7.4 containing 0.1 M NaCl and 0.1% Triton X-100 and 1 vol of fine glass powder by using a mortar and pestle. The crude extract then was sonicated for 20 sec. The homogenate was centrifuged at 3,000 × g for 5 min, and the resulting supernatant was stored at -20°C until further analysis.

6. Assay for Neutral Endopeptidase(NEP) Activity

NEP (EC 3.4.24.11) activity was determined by a coupled enzyme assay using the synthetic substrate glutaryl-Ala-Ala-Phe-4-methoxynaphthylamine[15].

The assay condition was optimized wherein the hydrolysis of the substrate is proportional to enzyme concentration and incubation time. The specificity of the enzyme activity was assessed in parallel experiments using phosphoramidon (1 μM), a potent inhibitor of NEP. The enzyme activity was expressed as nmol of product formed per min per mg of protein. Protein concentration of the cell-free extract was determined by the bicinchoninic acid method[16] using serum albumin as the standard.

7. Western Blot Analysis

The cell-free protein extract from the arthritic joints and nonarthritic joints prepared as above was used for the analysis of protein expression of cyclooxygenase 2 (Cox-2), IFN-γ, TNF-α, and the total IgG. For Western blot analysis, 40-50 μg of the protein was resolved over SDS/16% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk/1% Tween 20 in 20 mM Tris-buffered saline, pH 7.6) for 1 hr at room temperature, incubated with appropriate monoclonal primary antibody (for Cox-2, TNF-α and IFN-γ, Biosystem, USA) in blocking buffer for 1 hr to overnight at 4°C. Blots then were incubated with anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia) and detected by chemiluminescence using XAR-5 film. For the detection of total IgG, the secondary antibody was used directly on the blots. Blots were scanned by using PC-based scanning and analyzed by using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD), and the results were expressed in OD units normalized to β-actin expression.

8. CII ELISA

The CII-specific IgG2a in the joints and serum of arthritic mice was determined by an ELISA method[9] using CII. Briefly, 96-well microtiter plates were coated with CII overnight and washed with PBS, and then 100 μl of joint extracts diluted with PBS or various serum dilutions in PBS were applied to the wells, and the anti-CII antibodies were allowed to bind to the antigen. The wells were washed extensively, and then the alkaline phosphatase-conjugated goat anti-mouse IgG antibody (PharMingen) was applied, unbound antibody was washed, and the plates were developed by using the reagents of the mouse IgG isotype ELISA kit and the phosphatase substrate (Sigma, St. Louis, MO, USA). The plates were read in an ELISA reader at 410 nm, and the values were represented in arbitrary OD units.

9. Statistical analysis

Results were expressed as mean±SD. Statistical analysis was performed by Mann-Whitney U-test with P value < 0.05 for significance.

Ⅲ. Results and Discussion

DAE is widely used in the management and treatment of RA, particularly, in Korea. However, the mechanism by which DAE modify the clinical status of RA are not well understood.

According to a previous study, DAE inhibited production of IL-1β and TNF-α from macrophages in response to *in vivo* stimulation with bacterial lipopolysaccharides when the extract was administered into mice once a day for 7 days[12], suggesting that DAE administered orally into the patients inhibits cytokine production from both T cells and macrophages and effects on RA potently. Therefore, in this study, we examined the influence of DAE-fed on cellular immune responses by using CIA (collagen induced arthritis) mice, an experimental model for RA. The present results clearly demonstrated that the extract strongly inhibits T-cell activation including blastogenesis and cytokine production in response to antigenic stimulation *in vitro*. Furthermore, macrophages activation was also suppressed by the DAE. It was

observed that the DAE has significant reductive effects on the development of CIA in rats at dosages of 100-150 μg/kg/week. DAE treatment also suppressed the production of the proteases of cytoplasmic, lysosomal and matrix protease types[12].

DAE 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. However, biochemical and metabolic analysis of the constituents of DAE have to be analysed in further delineating its mechanisms of action in arthritis.

1. Incidence and Clinical Severity of Arthritis

In three independent experiments, 6-to 7-week-old DBA/1, male mice were used. In each experiment 12 mice were divided into two groups of six, and mice in one group (experimental) were fed DAE 1 week after arrival, while the animals of the second group (non-DAE-fed) were provided Phosphate Buffered Saline (PBS).

In two independent experiments, both groups of animals were immunized on day 10 with CII emulsified in CFA for the induction of arthritis[5,9]. Mice were boosted 3 weeks later with CII emulsified in incomplete Freund's adjuvant and then were observed for up to 40 days for the development of clinical arthritis. In the third experiment, the period of observation was extended to 85 days postimmunization with CII/CFA to differentiate between inhibition of the development of CIA or delayed onset of arthritis.

As summarized in Table 1, in experiment one, only two of the six mice in the DAE-fed group developed an atypical, mild inflammatory arthritis affecting either the toes or the metatarsophalangeal joints. In contrast, all six of the mice in the non-DAE-fed group developed the typical severe deforming arthritis involving the entire paw described earlier in the literature[3,9]. In the second experiment, three of the six mice in the DAE-fed group developed mild inflammatory arthritis similar to the arthritis observed in mice in experiment one. In experiment two, all six of the mice in the non-DAE-fed group developed the typical severe deforming arthritis within 40 days of immunization with CII. Mice in the third experiment were observed for up to 85 days after immunization with CII (Table 1). In this experiment, only two mice developed the mild clinical arthritis around day 40, and one mouse developed the severe arthritis in one joint on day 55 in the DAE-fed group. The remaining three mice showed no signs of clinical arthritis up to day 80 when the experiment was terminated. In this experiment, five of the six mice in the non-DAE-fed group developed severe arthritis in more than one joint between days 29 and 35 after immunization with CII whereas the sixth mouse remained arthritis free until the termination of the experiment on day 80. Thus the combined total incidence of arthritis in the DAE-fed group was 44% (8/18) whereas in the non-DAE-fed group the incidence of arthritis was 94% (17/18). In all of the experiments, in mice of the non-DAE-fed group the onset and progression of the disease was rapid, and the afflicted mice showed signs of loss of ambulation, whereas no loss of ambulation was noted in any animal of the DAE-fed group. In all three experiments the onset of arthritis was delayed in DAE-fed mice (range 37-39 days in experiment 1, 33-38 days in experiment 2, and 39-55 days in experiment 3). This finding was in contrast to the onset of arthritis in mice in the non-DAE-fed group where the onset of arthritis was early (range 28-37 days in experiment 1, 29-36 days in experiment 2, and 29-35 days in experiment 3). Additionally, in all of the cases where mice in the DAE-fed group developed clinical arthritis, usually only one paw was affected and the disease was not severe. This was in sharp contrast to the mice in the non-DAE-fed group where in all of the animals on the day of onset of arthritis (first day when clinical arthritis became apparent) two or more paws with pronounced edema and inflammation were observed. These results thus indicate that the DAE-fed mice, before disease-inducing immunization,

Table 1. Effect of DAE infusion on the incidence and severity of arthritis in DBA/1 mice

Group	No. Arthritic/ No. immunized	Arthritis index	Arthritic paws in each group	Mean day of onset (range)
Experiment 1				
DAE-fed	2/6 (33%)	1.05 ± 0.01	2	38 (37-39)
Non-DAE-fed	6/6 (100%)	4.22 ± 0.82	14	32 (28-37)
Experiment 2				
DAE-fed	3/6 (50%)	1.21 ± 0.03	3	36 (33-38)
Non-DAE-fed	6/6 (100%)	4.57 ± 0.65	16	32 (29-36)
Experiment 3				
DAE-fed	3/6 (50%)	1.43 ± 0.12	4	45 (39-55)
Non-DAE-fed	5/6 (84%)	4.10 ± 0.33	12	32 (29-35)
Overall				
DAE-fed	8/18 (44%)		9	
Non-DAE-fed	17/18 (94%)		42	

Mice were immunized intradermally in the tail with CII emulsified in CFA and boosted 3 weeks later via the same route with CII emulsified in incomplete Freund's adjuvant. Arthritis index was calculated by adding the total clinical severity score of each joint in each group of mice and dividing by the total number of arthritic mice in that group.

were less susceptible to the development of CIA in comparison to the non-DAE-fed mice; and the disease was also less severe in the DAE-fed mice as it was evident from the lower arthritis index in this group (Table 1).

2. Western Blot Analysis of Arthritic and Nonarthritic Joints

1) Cox-2 Level in the Joints

Cox exists in two isoforms and has different physiological functions primarily because of differences in its tissue expression and regulation[17]. Cox-1 is constitutively expressed in all tissues, and Cox-2 is inducible and tightly regulated. Expression of Cox-2 has been shown to be significantly up-regulated in inflammatory diseases, and in animal models it has been shown that this increased expression is correlated with prostaglandin production and inflammation. Cox-2 also is induced by proinflammatory cytokines such as TNF-α in many cell types, including synoviocytes, chondrocytes, and monocyte/macrophages[17]. Up-regulation of Cox-2 is considered to be involved in the pathological process as inhibitors of Cox-2 provide effective anti-inflammatory therapy and cause significant improvements in the signs and symptoms of RA[18]. This study is supported by our data that clearly show that the DAE-fed mice had mild arthritis or no arthritis, and this correlated with dramatically reduced amounts of Cox-2 protein in their joints (Fig. 1). This finding was in sharp contrast to arthritic mice in the non-DAE-fed groups where 2-fold higher amounts of Cox-2 were detected in all of the arthritic joints analyzed (Fig. 1, Table 2). Thus infusion of DAE appears to inhibit the production of Cox-2 in the joints of mice. Whether this inhibitory effect of DAE on Cox activity was directly mediated or indirectly mediated remains to be investigated.

Table 2. Western blot analysis of Cox-2 expression in arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice

	Non-DAE-fed (n=6)	DAE-fed (n=6)	P-value
	Mean±SD	Mean±SD	
Relative density of Cox-2 protein (normalized to β-actin)	1.55±0.13	0.60±0.08	0.000

statistical significance test was done by Mann-Whitney U-test

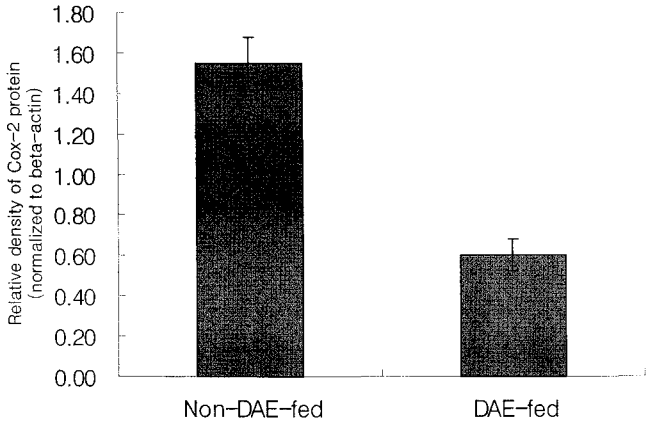

Fig. 1. Western blot analysis of Cox-2 expression in arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice
Error bars indicate SD.

2) TNF-α Level in the Joints

The DAE-fed mice had lower levels of TNF-α in the joints. TNF-α, a proinflammatory cytokine, is produced by activated macrophages and other cell types, and these cell types are abundant in the arthritic joints as has been shown in both the animal models and in RA patients. This abundance of TNF-α in arthritic joints provides evidence of its involvement in the disease pathology, which is supported by studies demonstrating that neutralization of TNF-α leads to decreased production of other inflammatory cytokines[19-20]. Further evidence for the involvement of TNF-α in destructive joint pathology also was obtained from studies in CIA in which it was shown that the administration of TNF-α to mice during the induction phase accelerated the onset of disease, and its blockade resulted in the reduction of severity of arthritis[21-22]. It subsequently was shown that treatment of arthritic mice with anti-TNF-α antibody can reduce clinical scores of the affected joints, paw swelling, and joint damage[23].

In the present studies, the DAE-fed mice had a significantly lower incidence of arthritis in comparison to the non-DAE-fed mice, and the mice that did develop arthritis in the DAE-fed groups had less inflammation and exhibited only mild clinical arthritis. In sharp contrast, 94% of the non-DAE-fed mice developed typical severe arthritis with pronounced edema and swelling of the affected joints (Table 3). Because in arthritic joints TNF-α is mainly produced by migratory inflammatory cells and has been convincingly demonstrated to be involved in joint inflammation in arthritis[19-23], we assayed for the presence of TNF-α protein in cell-free extracts of joints obtained from the non-DAE-fed

Table 3. Western blot analysis of TNF-α expression in arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice

	Non-DAE-fed (n=6)	DAE-fed (n=6)	P-value
	Mean±SD	Mean±SD	
Relative density of TNF-α protein (normalized to β-actin)	1.35±0.13	0.40±0.08	0.000

statistical significance test was done by Mann-Whitney U-test

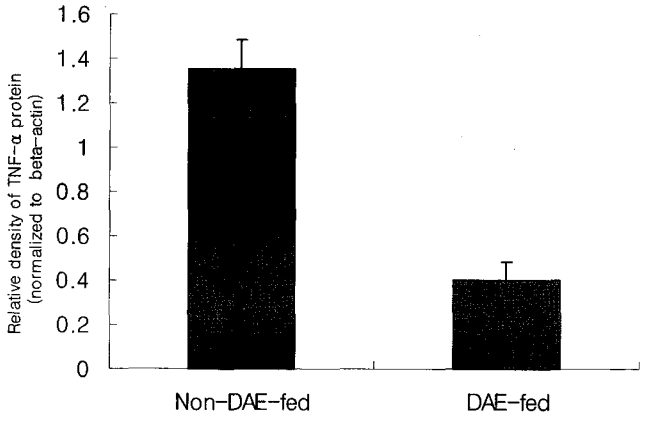

Fig. 2. Western blot analysis of TNF-α expression in arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice
Error bars indicate SD.

arthritic mice (severe inflammation and disease) and the DAE-fed arthritic mice (mild inflammation and disease). Results of Western blotting clearly show that levels of TNF-α protein were significantly reduced in the joints taken from the DAE-fed mice whereas in arthritic joints from the non-DAE-fed mice, markedly higher levels of TNF-α were present (Fig. 2).

3) IFN-γ Level in the Joints

The DAE-fed mice had lower IFN-γ levels and fewer IFN-γ-producing cells in arthritic joints. We investigated and quantified the frequency of cells producing the Th1 cytokine IFN-γ in the arthritic joints of the DAE-fed and non-DAE-fed mice. IFN-γ is produced by activated Th1-type T cells, whereas the Th2-type cells produce IL-4, IL-5, and IL-10 and provide help for B cell proliferation and differentiation[24]. IFN-γ induces activation of macrophages that produce a proinflammatory cytokine TNF-α and induces the expression of MHC class II, adhesion molecules, and several chemokines[25]. Previous studies have shown that severe CIA is associated with a strong Th1-type response with high levels of IFN-γ and absence of Th2-type cytokines IL-4 and IL-10[5,26]. Western blot analysis showed that the level of IFN-γ protein was markedly lower in the joints of DAE-fed mice in comparison to arthritic joints of non-DAE-fed mice that had about 5-fold higher amounts of IFN-γ present (Table 4, Fig. 3).

Table 4. Western blot analysis of INF-γ expression in arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice

	Non-DAE-fed (n=6)	DAE-fed (n=6)	P-value
	Mean±SD	Mean±SD	
Relative density of INF-γ protein (normalized to β-actin)	1.00±0.08	0.21±0.03	0.000

statistical significance test was done by Mann-Whitney U-test

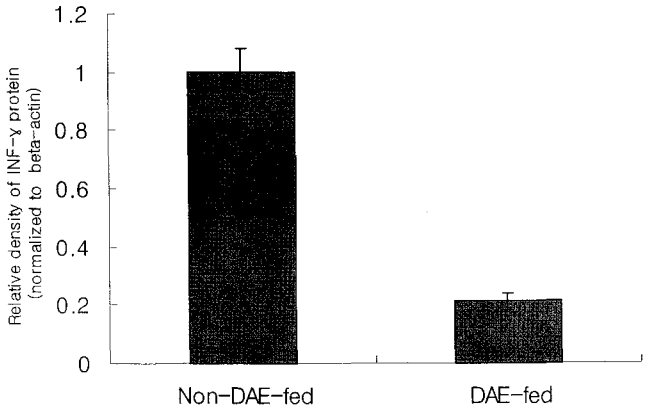

Fig. 3. Western blot analysis of INF-γ expression in arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice
Error bars indicate SD.

3. ELISA of CII-specific IgG antibody in the Joints

The DAE-fed mice had lower levels of total IgG antibody and CII-specific IgG antibody in the arthritic joints. Because the Th-1-type response (IFN-γ producing) is associated with the production of complement-fixing IgG2a antibodies, which are thought to bind to the cartilage and cause initial damage[4,8], we also determined the presence and level of total mouse IgG antibodies by Western blotting and CII-specific IgG2a antibodies by ELISA in the arthritic joints of DAE-fed mice and non-DAE-fed mice. The results showed that the level of total IgG antibodies in the arthritic joints of non-DAE-fed mice was markedly higher in comparison to the levels detected in the joints of DAE-fed mice. Results of CII-specific ELISA clearly show that concentration of CII-specific IgG2a antibodies in the cell-free extract prepared from the joints of DAE-fed arthritic mice was significantly less than that present in the cell-free extract prepared from the arthritic joints of the non-DAE-fed mice (Table 5, Fig. 4). Similar results were obtained when CII-specific IgG2a antibodies were measured in the serum that clearly showed that the titer of anti-CII-specific IgG2a antibodies was significantly higher in arthritic mice in the non-DAE-fed group in comparison to the mice in the DAE-fed groups. One possible mechanism for the reduction in the titer of pathogenic anti-CII antibodies could be an enhanced antioxidant defense system in the DAE-fed mice, but this hypothesis remains to be investigated.

Table 5. Titers of IgG2a antibodies reactive with CII in the arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice determined by ELISA method

	Non-DAE-fed(n=6)	DAE-fed(n=6)	P-value
	Mean±SD	Mean±SD	
O.D.	2.43±0.17	0.50±0.08	0.000

statistical significance test was done by Mann-Whitney U-test

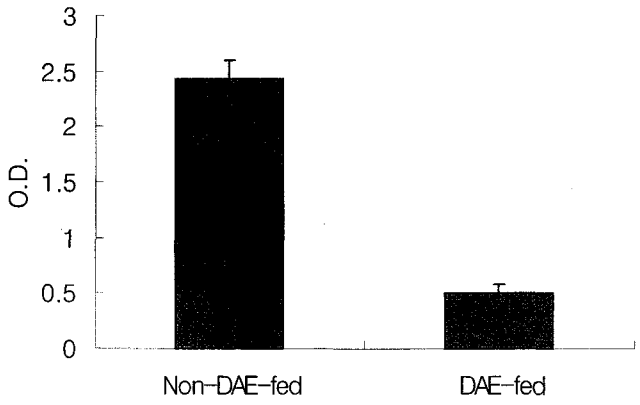

Fig. 4. Titers of IgG2a antibodies reactive with CII in the arthritic joints of non-DAE-fed and DAE-fed DBA/1 mice determined by ELISA method
Error bars indicate SD.

4. NEP activity in the joints

NEP inactivates the action of several biologically active peptides, including substance P[27]. NEP also has been shown to be expressed by migratory inflammatory cells[28] and thus may be a good marker for the cellularity of the arthritic joints. By using a synthetic peptide substrate, we determined the NEP activities of the cell-free extract of the joints from non-DAE-fed arthritic mice, nonimmunized mice (no arthritis), and DAE-fed mice with mild clinical arthritis. NEP activity was determined in the cell-free extracts of the joints prepared as described above. To estimate the basal level of enzyme activity, joints from the same lot of nonimmunized mice were used. The basal level of NEP activity in the cell-free extract of the nonarthritic joints of unimmunized mice was 162.50±16.84 nmol/min per mg of protein (mean± SD, n=6) (Table 6). In the joints of non-DAE-fed arthritic mice, NEP activity was increased by about 6-fold in comparison to the activity levels present in the nonarthritic joints of nonimmunized mice. In contrast, in the arthritic joints of DAE-fed mice NEP activity showed only 2-fold increase over the basal level (Table 6, Fig. 5). Thus DAE injection to mice apparently caused a significant attenuation in NEP activity in the arthritic joints of DBA/1 mice. The results are in accord with the view that the onset of arthritis is associated with an enhanced release of inflammatory mediator substances like substance P with a concomitant increase in neuropeptide inactivating enzyme, NEP. Chemical constituents of the DAE appear to attenuate the severity of arthritis by way of decreasing the cellular infiltration of the joints and the concentration of both the neuropeptide mediators and their rate of degradation in the synovium.

Table 6. Assay of NEP activity in the joints of control, non-DAE-fed and DAE-fed DBA/1 mice with CIA

	Control(n=6)	Non-DAE-fed(n=6)	DAE-fed(n=6)	P-value
	Mean±SD	Mean±SD	Mean±SD	
NEP activity (n moles substrate hydrolyzed/ min/mg protein)	162.50±16.84	944.75±51.87	332.00±35.67	0.000

statistical significance test was done by Mann-Whitney U-test

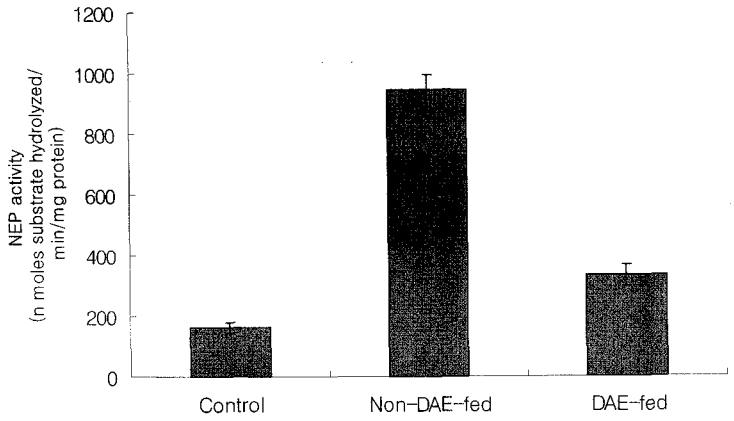

Fig. 5. Assay of NEP activity in the joints of control, non-DAE-fed and DAE-fed DBA/1 mice with CIA Error bars indicate SD.

In summary, our results suggest that DAE rich in antioxidants reduce the frequency of pathogenic Th1-type cells and associated pathogenic CII-specific IgG2a antibody in the affected joints. These joints also had significantly lower concentrations of inflammatory cytokines and other mediators of inflammation such as TNF-α and Cox-2.

Ⅳ. References

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