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# Anti-Arthritic and Analgesic Effect of NDI10218, a Standardized Extract of *Terminalia chebula*, on Arthritis and Pain Model

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## Abstract

The fruit of *Terminalia chebula* Retzius has been used as a panacea in India and Southeast Asia but its biological activities have not been fully elucidated. Here we report anti-arthritic and analgesic effect of NDI10218, a standardized ethanol extract of *Terminalia chebula*, on collagen-induced arthritis and acetic acid-induced writhing model, respectively. Arthritis was induced in DBA/1J mice by immunizing bovine type II collagen and mice were treated with NDI10218 daily for 5 weeks after the onset of the disease. NDI10218 reduced the arthritis index and blocked the synovial hyperplasia in a dose-dependent manner. The serum levels of pro-inflammatory cytokines TNF-$\alpha$, IL-6, and IL-1$\beta$ were significantly reduced in mice treated with NDI10218. Production of the inflammatory IL-17, but not immunosuppressive IL-10, was also inhibited in splenocytes isolated from NDI10218-treated arthritis mice. Administration of NDI10218 markedly decreased the number of T cell subpopulations in the regional lymph nodes of the arthritis mice. Finally, NDI10218 reduced the number of abdominal contractions in acetic acid-induced writhing model, suggesting an analgesic effect of this extract. Taken together, these results suggest that NDI10218 can be a new therapeutic candidate for the treatment of rheumatoid arthritis.

**Key Words:** *Terminalia chebula*, Arthritis, Pain, Anti-inflammatory, Analgesic

## INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation at multiple synovial joints, cartilage destruction, and bone erosion, which are manifested by joint swelling, stiffness, deformity, and severe pain (Feldmann *et al.*, 1996; Smolen and Steiner, 2003). Uncontrolled RA can cause systemic inflammation in multiple tissues such as lung and cardiovascular system, leading to comorbidities and increased mortality (Scott *et al.*, 2010). Although the etiology and pathogenesis mechanisms of RA are not fully understood, elevated levels of pro-inflammatory cytokines TNF-$\alpha$, IL-6 and IL-1$\beta$ are intimately associated with the progression and severity of the disease (Scott *et al.*, 2010). Recent studies revealed that IL-17 also contributes to chronic inflammation, cartilage and bone destruction in RA by inducing pro-inflammatory cytokines, matrix metalloproteinases, and osteoclast differentiation (Peck and Mellins, 2009). The presence of auto-antibodies known as Rheumatoid factor is believed to play important roles in initiating and maintaining inflammation by forming immune complexes and stimulating macrophages and synoviocytes to produce pro-inflammatory cytokines including TNF-$\alpha$. Infiltration of activated immune cells such as T cells, B cells, dendritic cells, and macrophages is frequently observed in inflamed synovial tissues, where these cells play central roles in the pathogenesis of RA by producing inflammatory cytokines and autoantibodies.

Conventional drugs used to treat RA patients include non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying antirheumatic drugs (DMARDs), and biological agents. NSAIDs and glucocorticoids had been used as first-line treatment due to their effects on alleviating pain and stiffness. However, their weak efficacy and inability to prevent joint damage or slow the disease progression have restricted their use to transient relief of the symptoms. DMARDs are widely used as standard RA therapy for their symptom-alleviating and disease-modifying effects, but they have adverse effects such as hepatotoxicity, blood dyscrasias, and intersti-

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tial lung disease (Scott *et al.*, 2010). Biological agents including TNF-$\alpha$ inhibitors are used in case of uncontrolled disease progression or severe side effects with other drugs, but high cost and opportunistic infection limit the prescription of biological agents. Due to various complications involved in long-term use of conventional medicines, many patients with arthritis are seeking alternative methods of disease management to improve their quality of life.

*Terminalia chebula* Retzius (Family: *Combretaceae*) is a widely growing evergreen tree in India and Southeast Asia. The fruit of *T. chebula* has been used extensively as an astringent, anti-tussive, anti-diarrheal, and anti-bleeding agent (Juang and Sheu, 2005). *T. chebula* has been shown anti-oxidant and cytoprotective activities in rat primary hepatocytes, liver, and kidney (Lee *et al.*, 2005a; Mahesh *et al.*, 2009), and protective effects against drug-induced gastric, intestinal, hepatic, and renal damages (Nadar and Pillai, 1989; Tasduq *et al.*, 2006; Bhattacharya *et al.*, 2007; Nariya *et al.*, 2009; Gopi *et al.*, 2010). Recent studies reported anti-inflammatory activities of *T. chebula* in systemic and local anaphylaxis and LPS-stimulated RAW264.7 cells (Shin *et al.*, 2001; Reddy and Reddanna, 2009).

In the present study, we investigated anti-arthritic and analgesic effects of NDI10218, a standardized ethanol extract of *T. chebula*, in mouse collagen-induced arthritis (CIA) and acetic acid-induced pain model, respectively, to evaluate NDI10218 as a new therapeutic option for the treatment of RA.

## MATERIALS AND METHODS

### Plant material and preparation of NDI10218

Dried ripe fruits of *T. chebula* were purchased from Bioland Co., Ltd. (Korea) and identified from their external appearance and histological anatomy by a scientist at the Institute of Traditional Medicine and Bioscience, Daejeon University. A voucher specimen was deposited in our laboratory (R&D center, BRN Science Co., Ltd., Seoul, Korea). NDI10218, the standardized extract of *T. chebula* fruits, was manufactured at Bioland Co., Ltd. In brief, the fruits of *T. chebula* were coarsely ground and the powder was extracted with 50% ethanol for 6 h at room temperature. After filtration, the extract was evaporated under reduced pressure and then powdered by vacuum drying. NDI10218 was standardized on the basis of chebulagic acid as indicative marker. Identification of NDI10218 was carried out by comparing the retention time of sample with that of authentic standards. Corilagin, chebulic acid, chebulinic acid, and punicalagin were purchased from Chromadex (Irvine, CA) and gallic acid was obtained from Sigma. Chebulagic acid was purified as described previously (Lee *et al.*, 2005b). An HPLC system (Agilent 1200) equipped with Shiseido Capcell Pack C18 4.6×150 mm column was used for analysis as previously described (Juang and Sheu, 2005). Aqueous phosphoric acid (0.1%) and acetonitrile mixture (80:20) was used as a mobile phase with a flow rate of 1 ml/min. The absorption peaks were detected at 216 nm and compared with standard marker molecules. The total chebulagic acid content in NDI10218 was found to be 4.62% (w/w).

### Collagen-induced arthritis (CIA)

All of the animal procedures were approved by the Experimental Animal Commission of the Institute of Traditional Medicine and Bioscience at Daejeon University. Female DBA/1J mice (6 weeks old; Harlan, San Jose, CA, USA) were housed (three mice/cage) and given water *ad libitum*, with a 12 h light-dark cycle beginning at 7:00 a.m. After acclimation for one week, mice were received 100 $\mu$g of bovine type II collagen (Sigma) in Complete Freund's Adjuvant (Sigma) by subcutaneous injection at the tail on day 0 and a booster injection was done on day 21 with the same antigen in Incomplete Freund's adjuvant. Normal non-immunized mice were used as negative controls. Mice were monitored for signs of arthritis, and each paw was scored individually as follows: 0=normal, 1=slight erythema, 2=slight edema, 3=increased edema with loss of landmarks, 4=marked edema, and 5=marked edema with ankylosis on flexion. Each mouse was assigned an arthritis score (articular index) that equaled the sum of the score for each paw, so that the possible maximum score per mouse was 20. Two weeks after the booster injection, mice were randomly assigned to one of the five treatment groups (n=6). Each groups were orally administered with either vehicle (0.5% methylcellulose), NDI10218 (62.5, 125, or 250 mg/kg dissolved in 0.5% methylcellulose), or 2 mg/kg methotrexate daily for 5 weeks. Severity of arthritis was scored every week. On the final day, blood was collected by cardiac puncture, and the knee joints from the hind limbs, regional draining lymph nodes, and the spleen were obtained.

### Histological analysis

For histologic analysis of the knee joints, the hind limbs of mice were removed and fixed in 10% neutral buffered formalin, decalcified in 5% formic acid, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome (M-T).

### Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1$\beta$, IL-6, TNF-$\alpha$, and IFN-$\gamma$ in serum were measured using ELISA kits from BioSource (Camarillo, CA) according to manufacturer's instructions. For splenocyte culture, cells were isolated by chopping the spleen and removing the tissue debris through mesh screen and red blood cells were lysed in lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA). Splenocytes were seeded at $2\times10^5$ cells/well in 96-well plate and stimulated with type II collagen (1 $\mu$g/ml) for 48 h. Culture supernatants were harvested and the amounts of IL-10 and IL-17 were measured as described above using kits from BioSource. Experiments were done in triplicate and data represent mean ± standard error of mean from 6 mice.

### Flow cytometry

Cells were obtained from the synovial tissues of the knee joints and draining lymph nodes as described previously (Lee *et al.*, 2005b). Briefly, the synovial tissue was isolated after removing the surrounding muscle, patellar ligament, and patella. Care was taken not to damage local blood vessels. The tissue was cut into small pieces and incubated with 1 $\mu$g/ml type VI collagenase (Sigma) in Hanks' balanced salt solution with 2% FBS and 1 mM EDTA. Cells were harvested, washed, and incubated with antibodies for flow cytometry. For draining lymph nodes, the inguinal and mesenteric lymph nodes were removed, cut into pieces, and passed through mesh screen to obtain single cell suspension. Cells were harvested, washed, counted, and incubated with antibodies for 30 min at 4°C. PE-anti-CD3e, FITC-anti-CD4, FITC-anti-CD8, PE-anti-CD25,

FITC-anti-CD69, PE-anti-Gr-1, and FITC-anti-CD11b were purchased from Becton Dickinson (BD) PharMingen (San Diego, CA). Cells were analyzed on FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

### Acetic acid-induced writhing test

All experimental procedures were approved by the Seoul National University Animal Experiment Ethics Committee. Thirty-two male ICR mice (7 week-old) were obtained from Central Laboratory Animals (Seoul, Korea). All mice were housed (four mice/cage) and given water *ad libitum*, with a 12 h light-dark cycle beginning at 7:00 a.m. After acclimation for one week, mice were randomly assigned to one of the four treatment groups (n=8). Vehicle, aceclofenac (10 mg/kg), and NDI10218 (30, 100 and 300 mg/kg) were orally administered. An hour after the delivery, 0.2 ml of acetic acid (1.2%, w/v) in saline was injected intraperitoneally. The numbers of abdominal writhing movements were recorded for 20 min starting 5 min after intraperitoneal injection in each animal.

### Statistical analysis

Statistical analyses were performed by ANOVA test with Turkey's post hoc analysis. Statistical significance was set at **p*<0.05, ***p*<0.01, ****p*<0.001.

## RESULTS

### The fingerprint of NDI10218 established by high performance liquid chromatography (HPLC)

The fingerprint of NDI10218, the standardized ethanol extract of *T. chebula*, was obtained by HPLC (Fig. 1). Based on the chromatogram of the extract, six main component peaks were detected: chebulic acid (retention time: 5.20 min), gallic acid (retention time: 8.37 min), punicalagin (retention time: 27.52 min), corilagin (retention time: 39.06 min), chebulagic acid (retention time: 50.72 min), and chebulinic acid (retention time: 61.40 min).

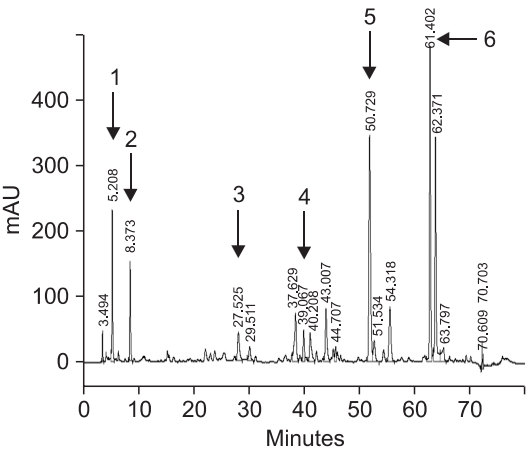


**Fig. 1.** The HPLC chromatogram of NDI10218. Key to peak identity: 1, Chebulic acid: 2, Gallic acid: 3, Punicalagin: 4, Corilagin: 5, Chebulagic acid: 6, Chebulinic acid.

### Anti-arthritic activity of NDI10218 on the clinical characteristics of CIA

The effect of NDI10218 on the incidence and severity of arthritis was evaluated by scoring the arthritis index every week in CIA mice. Arthritis index was increased in vehicle-treated control mice with time, while it was retarded significantly in mice treated with NDI10218 at 62.5 mg/kg from 4 weeks after the treatment (Fig. 2). Administration of NDI10218 at 125 mg/kg and 250 mg/kg alleviated the severity of the disease from 2 weeks after administration similar to methotrexate at 2 mg/kg, the most widely used anti-rheumatic drug with disease modifying activity. These data demonstrate anti-arthritic effect of NDI10218 in mouse CIA model.

Administration of NDI10218 did not show any effect on the body weight nor elicit behavioral change in mice (data not shown), suggesting that NDI10218 is not toxic *in vivo* at concentrations used in this experiment. We did not check the LD50 value in normal mice nor the mice with CIA. But we found that the LD50 in Sprague-Dawley rat was 5,209 mg/kg as a single dose. We found no toxicity up to 2,000 mg/kg/day with repeated oral administration for 2 weeks in SD rats. If we convert these values to mouse equivalent doses according to the US FDA CDER (Center for Drug Evaluation and Research)'s guideline based on the body surface area, 5,209 mg/kg in rat corresponds to 10,418 mg/kg in mouse and 2,000 mg/kg/day in rat corresponds to 4,000 mg/kg/day in mouse. Since we used 250 mg/kg as a maximum dose in mouse CIA model, we believe this concentration is far below the extrapolated LD50 value in mice.

### Histological analysis of the knee joints

To examine the protective effect of NDI10218 on the destruction of articular joints in CIA mice, sections of the hind knee joints were prepared from normal and CIA mice (Fig. 3). Histological examination of the synovial joints of the vehicle-

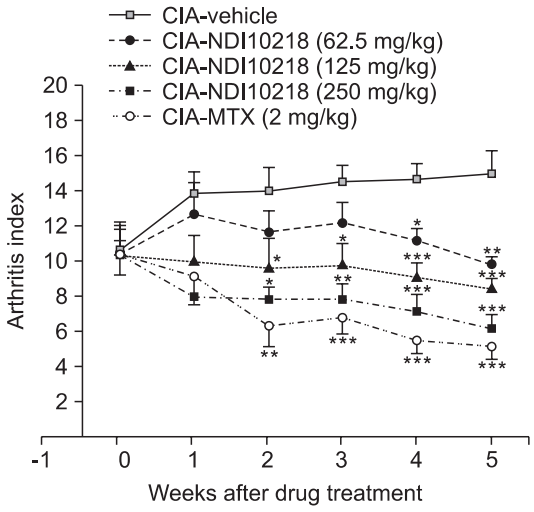


**Fig. 2.** Effect of NDI10218 on the progression of collagen-induced arthritis. Two weeks after booster injection, mice were divided into 5 groups and orally administered with vehicle, indicated amounts of NDI10218, or methotrexate for 5 weeks. Disease progression was monitored by scoring arthritis index every week. Data represent mean ± S.E.M. (n=6). **p*<0.05, ***p*<0.01, and ****p*<0.001 as compared with vehicle-treated CIA mice.

treated CIA mice revealed extensive cellular infiltration, synovial hyperplasia, and joint narrowing (Fig. 3B). Severe pannus formation and focal erosions of the cartilage and bone in the area of direct pannus invasion were also observed in the synovial joints of vehicle-treated mice (Fig. 3B) as compared with those of normal mice (Fig. 3A). These pathological changes were reduced markedly in NDI10218 or methotrexate-treated mice (Fig. 3C-F), and the protective effects were obvious even in mice treated with as little as 62.5 mg/kg of NDI10218.

### Inhibitory effect of NDI10218 on the production of pro-inflammatory cytokines

To understand the anti-arthritic mechanisms of NDI10218, we measured the concentrations of cytokines TNF-α, IL-6, IL-1β, and IFN-γ in the serum of normal and CIA mice. Consistent with the arthritis index and severe histological lesions, pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were systemically increased in the sera of vehicle-treated CIA mice (Fig. 4A-C). Treatment of NDI10218 suppressed the production of TNF-α and IL-6 in a dose-dependent manner. NDI10218 at doses of 125 mg/kg and 250 mg/kg reduced the serum levels of TNF-α and IL-6 significantly (Fig. 4A, B). Marked inhibition of IL-1β production was observed at all doses of NDI10218, and the serum level of IL-1β in the NDI10218-treated mice was similar to one in normal mice (Fig. 4C). On the contrary, administration of NDI10218 did not affect the production of IFN-γ in CIA mice even at 250 mg/kg (Fig. 4D), demonstrating that the suppressive effect of NDI10218 on cytokine production is specific to pro-inflammatory cytokines.

To check the influence of NDI10218 on the production of

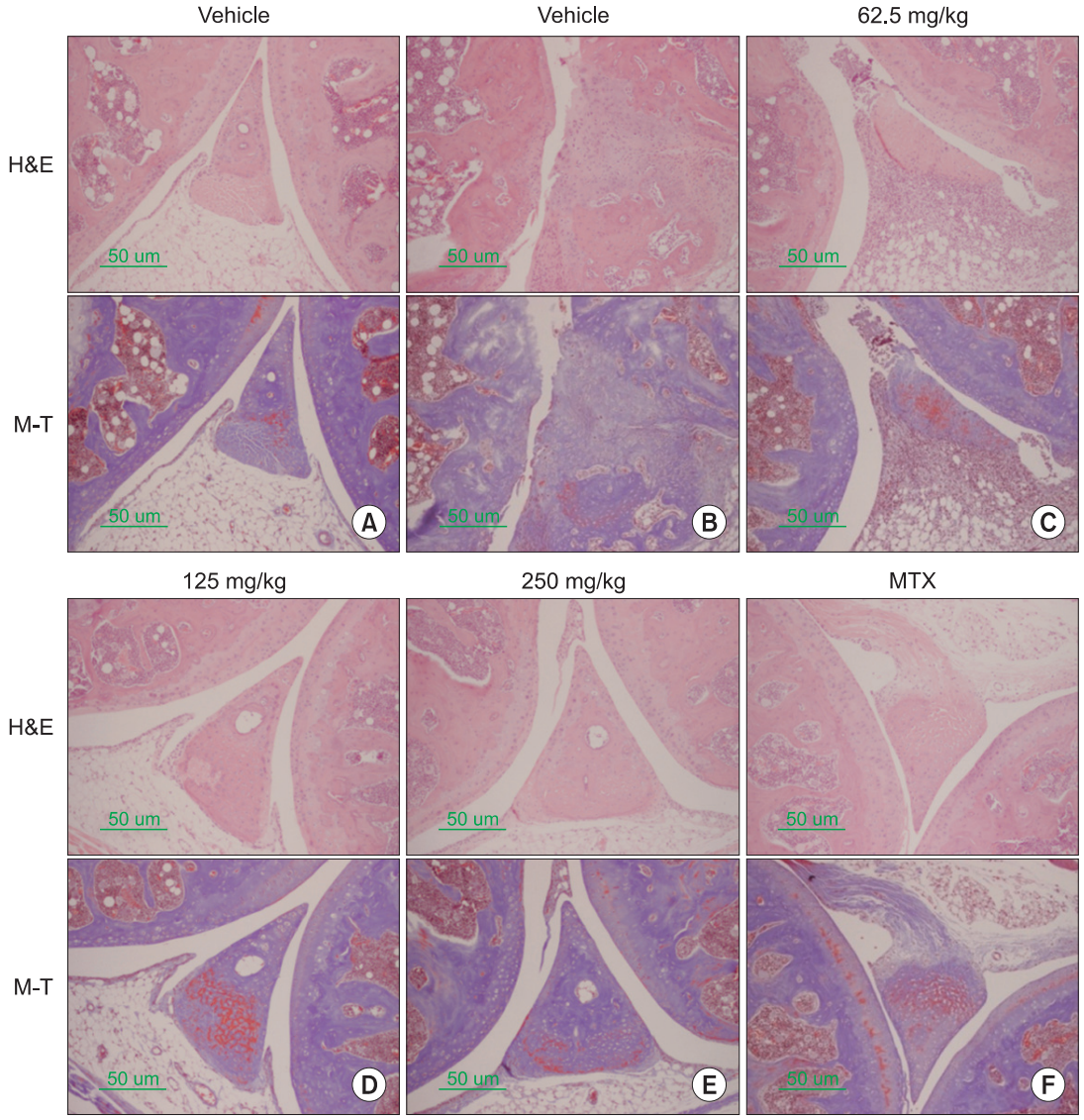


**Fig. 3.** Histological analysis of the effect of NDI10218 on mouse CIA. Sections from hind knee joints were obtained and stained with either hematoxylin-eosin (H&E) or Masson's trichrome (M-T). Representative histological sections from normal mice (A), vehicle-treated CIA mice (B), CIA mice treated with indicated amounts of NDI10218 (C-E), and methotrexate-treated CIA mice (F) were shown. Bars represent 50 μm.

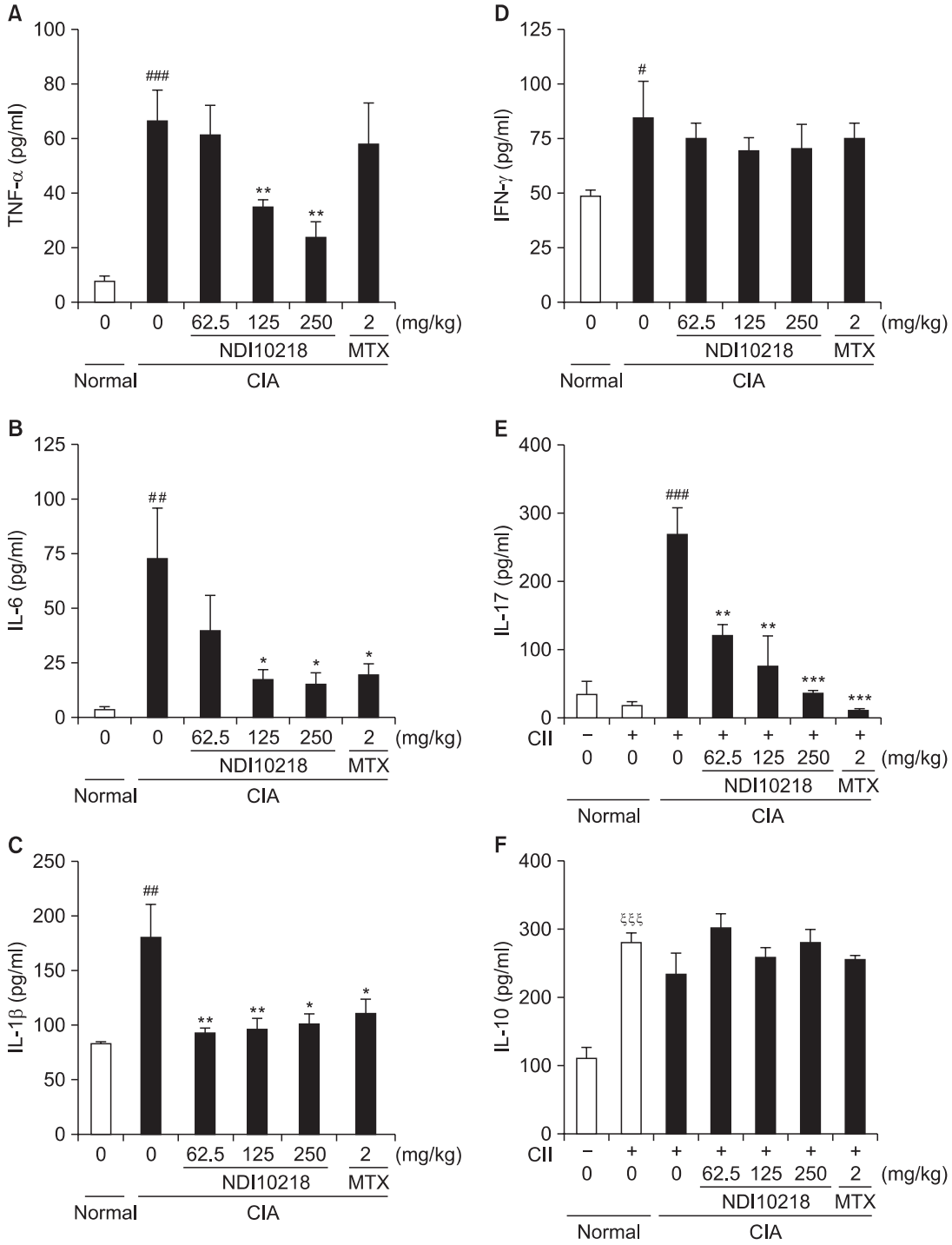


**Fig. 4.** NDI10218 reduced the production of pro-inflammatory cytokines. Blood was obtained from normal (open bar) or CIA mice (closed bar) on the last day of experiment and the levels of TNF-α (A), IL-6 (B), IL-1β (C), and IFN-γ (D) in the sera were measured by ELISA. Splenocytes were obtained from normal (open bar) or CIA mice (closed bar), and plated in 96-well plates. Cells were grown in the presence or absence of type II collagen (1 μg/ml) for 48 h. The amounts of IL-17 (E) and IL-10 (F) in the culture supernatant were measured by ELISA. Data represent mean ± S.E.M. (n=6). $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ as compared with vehicle-treated normal mice; $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ as compared with vehicle-treated CIA mice. $^{\xi\xi\xi}p<0.001$ as compared with untreated splenocytes from normal mice.

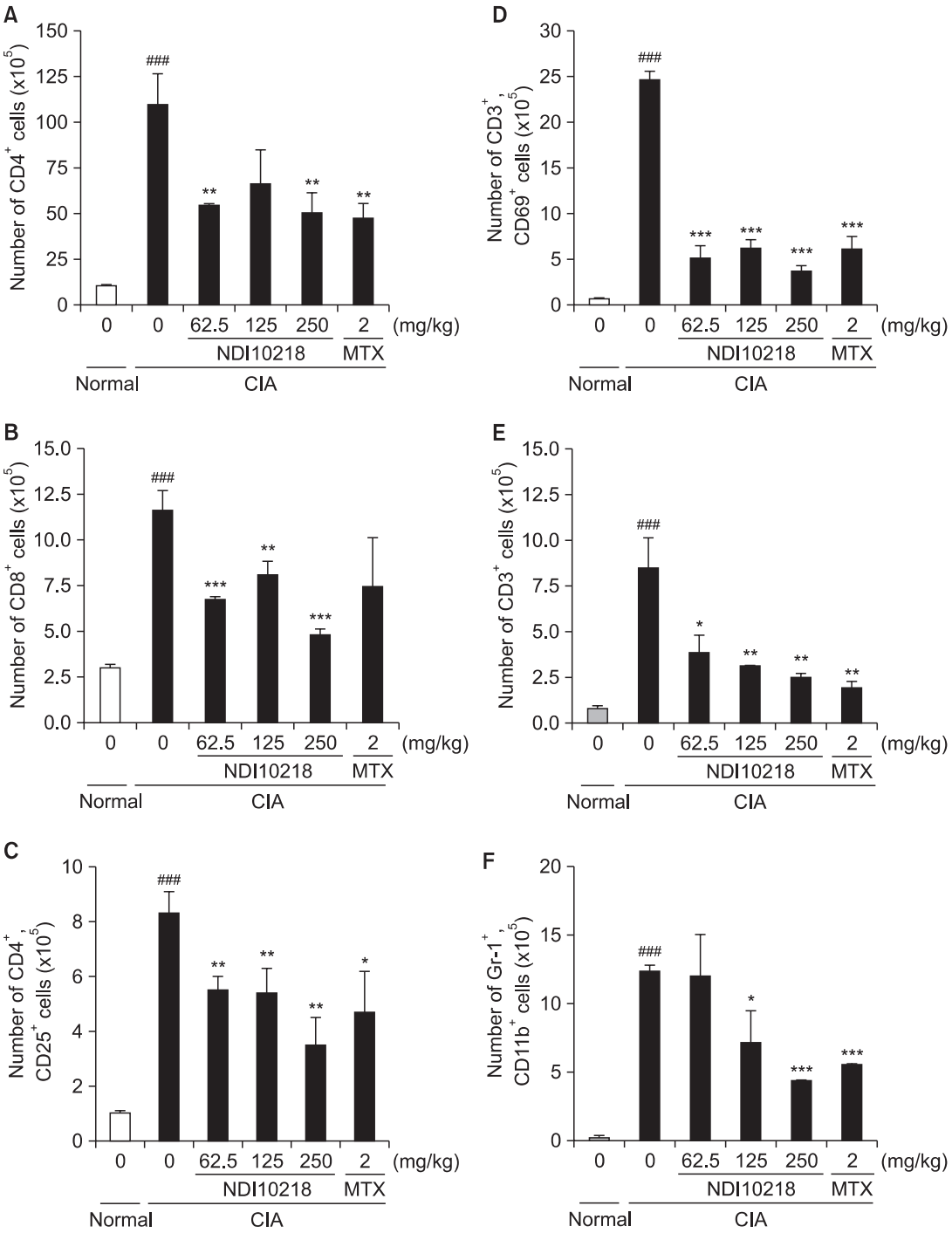


**Fig. 5.** NDI10218 suppressed the accumulation of immune cells in the draining lymph nodes and joints in CIA mice. Cells were isolated from the inguinal and mesenteric lymph nodes (A-D) or from the knee joints (E-F) of the normal (open bar) or CIA mice (closed bar), and stained with specific antibodies. The numbers of helper T cells (A), cytotoxic T cells (B), activated T cells (C), early activated T cells (D), $CD3^+$ T cells (E), and granulocytes (F) were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Percentage of each cell type was obtained and converted to an absolute cell number using total immune cell number in the draining lymph nodes or knee joints. Data represent mean ± S.E.M. (n=6). $^{###}p<0.001$ as compared with vehicle-treated normal mice; $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ as compared with vehicle-treated CIA mice.

IL-17 and IL-10, splenocytes were isolated from normal and CIA mice and seeded in 96-well plates in triplicate. Cells were stimulated with type II collagen and induction of IL-17 and IL-10 in the culture supernatant was measured by ELISA. Splenocytes from vehicle-treated CIA mice produced significant amount of IL-17 upon stimulation with collagen compared with cells from normal mice (Fig. 4E). Production of inflammatory cytokine IL-17 was reduced in splenocytes isolated from NDI10218-treated mice in a dose dependent manner, and the level of IL-17 from cells derived from mice treated with NDI10218 at 250 mg/kg was equal to that from unstimulated cells derived from normal mice.

Production of anti-inflammatory cytokine IL-10 was increased in splenocytes isolated from normal mice upon addition of collagen, similar to the increase of IL-10 in cells from vehicle-treated CIA mice (Fig. 4F). The amounts of IL-10 produced from splenocytes of NDI10218-treated mice were not different significantly from those from splenocytes of vehicle-treated CIA mice, demonstrating that NDI10218 has no effect on the production of anti-inflammatory cytokine IL-10.

### Inhibitory effects of NDI10218 on the accumulation of immune cells in the draining lymph nodes and knee joints

To dissect the anti-arthritic effect of NDI10218 in detail, we measured the number of total immune cells in the draining lymph nodes and knee joints by hemocytometer and analyzed the percentage of each cell type by flow cytometry. Representative dot plot results showing the percentage of each cell type were presented as Supplementary Fig. 1. The absolute number of each cell type was obtained by multiplying total cell number with the percentage of each cell type obtained from flow cytometry. The number of helper T cells (Fig. 5A), cytotoxic T cells (Fig. 5B), activated helper T cells (Fig. 5C), and activated T cells (Fig. 5D) in the draining lymph nodes was increased dramatically in vehicle-treated CIA mice. Administration of NDI10218 reduced the number of these cells dramatically at all doses examined in CIA mice (Fig. 5A-D), suggesting inhibitory roles of NDI10218 on the proliferation and activation of T cells in the regional lymph nodes.

A similar inhibitory effect of NDI10218 on the recruitment of immune cells was observed in the knee joints where the number of T cells (Fig. 5E) and granulocytes (Fig. 5F) was decreased in a dose-dependent manner, in consistent with histological examination of the knee joint (Fig. 3C-E).

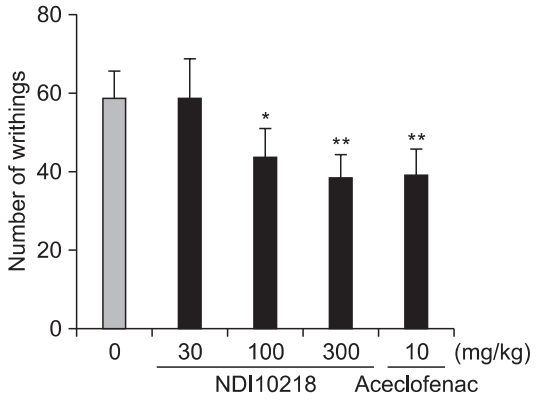


**Fig. 6.** Effect of NDI10218 on the relief of pain in acetic acid-induced writhing model. Mice were treated with indicated amounts of NDI10218 1 hr before the administration of acetic acid. The number of abdominal contraction was measured for 20 min after injection of acetic acid. Data represent mean ± S.E.M. (n=8). **p*<0.05 and ***p*<0.01 as compared with vehicle-treated mice.

### Acetic acid-induced writhing in mice

To check if NDI10218 could alleviate the joint pain occurred in RA patients, we examined the *in vivo* analgesic activity of NDI10210 in acetic acid-induced writhing model. Intraperitoneal administration of acetic acid induced 58.6 ± 7.1 writhes in control mice during the 20 min observation period. However, the mice that had received NDI10218 at doses of 30, 100 and 300 mg/kg writhed 58.4 ± 10.2, 43.7 ± 7.2, and 38.5 ± 5.8 times, respectively (Fig. 6), showing a significant analgesic effect at doses 100 and 300 mg/kg of NDI10218.

## DISCUSSION

Here we examined the anti-arthritic effect of NDI10218 in mouse CIA model since type II collagen is the main constituent of articular cartilage and CIA model in arthritis susceptible DBA/1J mice has been used extensively in the study of RA due to its similarity to human RA both immunologically and pathologically (Kannan *et al.*, 2005). Our study demonstrated that oral administration of NDI10218 efficiently alleviated CIA in mice, as evidenced by significant reduction in arthritis index (Fig. 2). Synovial inflammation and bone and cartilage erosion was suppressed markedly in the histological lesions of the CIA mice treated with NDI10218, further demonstrating its anti-arthritic effect (Fig. 3). Serum levels of pro-inflammatory cytokines TNF-$\alpha$, IL-6, and IL-1$\beta$ were reduced significantly (Fig. 4A-C), and the production of IL-17 from isolated splenocytes was also inhibited in a dose-dependent manner in NDI10218-treated CIA groups (Fig. 4E). TNF-$\alpha$ plays central roles in the pathogenesis RA through initiating synovial inflammation and joint destruction (Scott *et al.*, 2010). TNF-$\alpha$ augments synovial inflammation by driving overproduction of proinflammatory cytokines IL-6 and IL-1$\beta$. Cytokines TNF-$\alpha$, IL-1$\beta$, and IL-17 induce joint destruction by activating osteoclasts through increased RANKL expression on synovial fibroblast and by stimulating chondrocytes to produce matrix metalloproteinases (van den Berg *et al.*, 2007). Taken together, the anti-inflammatory capability of NDI10218 in reducing the levels of TNF-$\alpha$, IL-6, IL-1$\beta$, and IL-17 might explain the anti-arthritic effect of NDI10218.

NDI10218 also decreased the number of T cells and activated T cells in the regional lymph nodes, and blocked the recruitment of T cells and granulocytes to the affected synovial joints (Fig. 5). These results indicate immunosuppressive effect of NDI10218 on T cell proliferation and activation and on the recruitment of immune cells to the affected sites.

Finally, we showed analgesic effect of NDI10218 in acetic acid-induced pain model (Fig. 6). Since acetic acid-induced writhing is used as a general model for peripheral and central pains (Le Bars *et al.*, 2001), further studies including tail-flick test or thermal hyperalgesia would be necessary to narrow down the mechanism of action of NDI10218.

RA is a complex refractory autoimmune disease characterized by articular pain, polyarthritis, and joint destruction. RA results from diverse pathogenic events including systemic and local inflammation, cellular and humoral immune responses,

extensive angiogenesis, and destruction of bone and cartilage by osteoclasts and matrix metalloproteinases (Smolen and Steiner, 2003). Due to the complexity of the disease, combination therapy interfering multiple pathogenic events or target proteins is preferred. We showed here that NDI10218 suppressed inflammatory responses significantly at multiple levels: production of pro-inflammatory cytokines, activation and proliferation of lymphocytes in the regional lymph nodes, and infiltration of immune cells in the affected joints. Histological analysis revealed cartilage and joint destruction was blocked efficiently in NDI10218-treated groups. The protective effect of NDI10218 on joint destruction may result partly from its anti-inflammatory activity, and partly from its effect on the inhibition of matrix metalloproteinases as previously reported (Kumar *et al.*, 2008; Manosroi *et al.*, 2010). We also showed for the first time the analgesic effect of NDI10210. Taken together, our results clearly demonstrated *in vivo* anti-arthritic and underlying immunomodulatory effects at multiple levels. Our finding provides a firm basis for the use of NDI10218 in alleviating and improving RA in the future.

*T. chebula* is famous for containing high levels of hydrolysable tannins such as chebulagic acid, chebulinic acid, and gallic acid (Fig. 1)(Juang and Sheu, 2005). Although the biological activities of these constituents are not clearly understood, some of which have shown anti-inflammatory activities. Gallic acid and chebulagic acid isolated from *T. chebula* were reported to inhibit cytotoxic T cell-mediated cytotoxicity (Manosroi *et al.* 2010), and chebulagic acid suppressed the expression of TNF-$\alpha$ and IL-6 by inhibiting NF-$\kappa$B activation and MAPK phosphorylation in LPS-stimulated RAW 264.7 macrophages (Reddy and Reddanna, 2009). Recently, IL-17 has emerged as a key driver in the pathogenesis of RA, and its importance has been proven in animal models and clinical trials where neutralization of IL-17 ameliorated the disease significantly (Hamada *et al.*, 1997; Lubberts *et al.*, 2004; Genovese *et al.,* 2010). Here we found the inhibitory effect of NDI10218 on the production of IL-17 for the first time. It remains to be studied which component of NDI10218 is responsible for the inhibition of IL-17.

Regulatory T cells are a subset of $CD4^+CD25^+Foxp3^+$ T cells that suppress the proliferation of responder T cells and the expression of proinflammatory cytokines (Notley and Ehrenstein, 2010). Due to their anti-inflammatory roles, induction of regulatory T cells from patients with autoimmune diseases was considered as a good therapeutic strategy. However, recent findings showed that the regulatory T cells from patients with RA are defective in suppressing the expression of proinflammatory cytokines from activated T cells and monocytes (Ehrenstein *et al.*, 2004). Thus, more emphasis has been putting on enhancing or restoring the function of regulatory T cells rather than inducing regulatory T cells. Chebulagic acid, one of the major constituents of NDI10218, was reported to inhibit the onset and progression of CIA by inducing regulatory T cells (Lee *et al.*, 2005b). We found that NDI10218 was also efficient in inducing regulatory T cells as judged by the increased expression of Foxp3, one of the regulatory T cell markers (data not shown). It is intriguing to study whether NDI10218 or chebulagic acid could also enhance the function of regulatory T cells in CIA model. NDI10218 contains high levels of tannins with anti-oxidant and anti-inflammatory activities including chebulagic acid, and is able to inhibit the production of IL-17, which has not been reported with chebulagic acid. Although chebulagic acid could be considered as a lead compound for the development of RA drug, the value of NDI10218 as an alternative or complementary medicine should not be underestimated.

Methotrexate has been used as a first-line therapy for RA as a single or in combination with other drugs, but has toxicities including ulcerative stomatitis, nausea, leukopenia, and abdominal distress (Furst, 1997). NSAIDs also have gastrointestinal and cardiac toxicities, limiting the use of these drugs in the treatment of RA. Increased risk of opportunistic infections and cancer has been reported with prolonged use of biological agents. Therefore, the needs are growing for the development of agents that are capable of ameliorating RA with limited side effects. *T. chebula* has been widely used for wounds and ulcers and shown anti-oxidant (Lee *et al.*, 2005a; Mahesh *et al.*, 2009), anti-ulcer (Nadar and Pillai, 1989; Bhattacharya *et al.*, 2007), hepato- and nephro-protective (Tasduq *et al.*, 2006; Gopi *et al.*, 2010), and wound healing activities (Suguna *et al.*, 2002; Kumar *et al.*, 2008). Furthermore, recent report demonstrated enteroprotective effect of *T. chebula* on methotrexate-induced intestinal damage in rat (Nariya *et al.*, 2009). It also has been shown to possess anti-carcinogenic (Kaur *et al.*, 1998; Saleem *et al.*, 2002), anti-bacterial (Sato *et al.*, 1997; Ahmad *et al.*, 1998; Malekzadeh *et al.*, 2001; Suguna *et al.*, 2002), anti-fungal (Vonshak *et al.*, 2003), and anti-viral activities (Badmaev and Nowakowski, 2000; Ahn *et al.*, 2002). The multifaceted roles of *T. chebula* in ulcer, infection, and cancer might offer a useful therapeutic opportunity for RA as an alternative or complementary medicine to current RA therapeutics.

## CONFLICT OF INTEREST

J.B.S., E.M.J., J.Y.P, S.S.C., E.W.C., D.S.S., D.Y.P., and T.G.L are employees of, and stockholders of BRN Science Co., Ltd. J.Y.J. is a former employee of BRN Science Co., Ltd. The remaining authors declare no conflict of interest.

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