

The Preventive Effects of Nanopowdered Peanut Sprout-added Caciocavallo Cheese on Collagen-induced Arthritic Mice

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Abstract

The present study was carried out to investigate the effects of nanopowdered peanut sprout-added Caciocavallo cheese (NPCC) on the prevention and treatment of rheumatoid arthritis in DBA/1J mice immunized with type II collagen. After the induction of arthritis, the mice were being divided into five groups: (1) normal, no immunization; (2) CIA, collagen-induced arthritis; (3) MTX, collagen-induced arthritis treated with methotrexate (0.3 mg/kg body weight); (4) CC, collagen-induced arthritis treated with Caciocavallo cheese (0.6 g/d); (5) NPCC, collagen-induced arthritis treated with nanopowdered peanut sprout-added Caciocavallo cheese (0.6 g/d). Nanopowdered peanut sprout was ranged from 300 to 350 nm, while regular powdered peanut sprouts were ranged from 50 to 150 μ m. The NPCC group had considerable reductions of clinical scores and paw thicknesses at the end of experiment as compared to the CIA group. In the serum analysis, the TNF- α , IL-1 β , IL-6 and IgG₁ levels in the NPCC group have decreased by 69.4, 75.9, 66.6, and 61.9%, respectively, when compared to the CIA group. The histological score and spleen index of the NPCC group were significantly lower than the CIA group. In conclusion, the feeding NPCC method could delay and/or prevent the rheumatoid arthritis in the collagen-induced arthritis mouse model. Based on this study, nanopowdered peanut sprouts could be applied to various functional cheeses.

Key words: rheumatoid arthritis, nanopowdered peanut sprout, Caciocavallo cheese

Introduction

Rheumatoid arthritis is a systemic autoimmune illness characterized by chronic inflammation of synovial joints leading to destruction of cartilage and bone, and affects about 1% of world's total population (Borchers *et al.*, 2004; Chunxia *et al.*, 2011; Maini and Taylor, 2000; Sanchez-Fidalgo *et al.*, 2010; Stamp *et al.*, 2005). A great number of patients suffering from rheumatoid arthritis have been looking for alternative or complementary therapies of which diet is one of the most important factors.

Nowadays, peanut sprouts are obtained from the germination of peanut kernels, and have usually been utilized in the diet as a health food in Asia (Lee *et al.*, 2013; Xiong *et al.*, 2014). According to Burns *et al.* (2002) and Xiong *et al.* (2014), peanut sprouts are rich in flavonoid and resveratrol which can contribute to disease preventive and health promoting properties, such as various cancers,

diabetes mellitus, aging, cardiovascular disease, and so on. Wang *et al.* (2005) reported that the resveratrol contents of the sprouts germinated from a variety of peanuts ranged from 11.7 to 25.7 μ g/g. In particular, Elmani *et al.* (2006) investigated the effects of resveratrol on arthritis in the rabbit model. According to them, decreased cartilage destruction was determined after injecting 10 μ mol/kg resveratrol in DMSO into the right knees of the rabbit. Recently, Xuzhu *et al.* (2013) found that after 23 d treatment of resveratrol (20 mg/kg), the collagen-induced arthritis in the mice was significantly reduced when they evaluated the disease incidence, number of involved paws, footpad thickness, and clinical index.

In recent years, nanotechnology has captured the attention of researchers, manufacturers, and the general population (Park *et al.*, 2007; Rasenack and Muller, 2004). According to Park *et al.* (2007), nanocalcium supplementation in milk may be an effective way to improve bone calcium metabolism for ovariectomized rats. Due to the advantage of size effect and high surface reactivity of nanoparticles, nanotechnology is already used in pharmaceutical applications to increase the bioavailability of drugs. However, there are a few reports on applications of

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nanotechnology in food industry, especially in dairy products. For example, in our previous study (Seo *et al.*, 2009), we investigated the physicochemical, microbial, and sensory properties of yogurt supplemented with nanopowdered chitosan during storage.

Caciocavallo cheese is a semi-hard cheese and is originally manufactured in the Palermo province (Sicily, Italy). So-called healthy foods, particularly those with nutraceutical properties, are in great demand in our health conscious society. Nutraceutical Caciocavallo cheese could be a good vehicle in this respect if nutraceutical ingredients, such as nanopowdered peanut sprouts, were to be added into the cheese. However, there is no report in the literature on the production of nanopowdered peanut sprouts-added Caciocavallo cheese. Furthermore, the effects of Caciocavallo cheese or nanopowdered peanut sprouts-added Caciocavallo cheese on prevention and treatment of rheumatoid arthritis have not been examined yet. Therefore, the objective of the present study was to elucidate the effects of nanopowdered peanut sprouts-added Caciocavallo cheese on the reduction of rheumatoid arthritis in DBA/1J mice immunized with type II collagen.

Materials and Methods

Materials

Powdered peanut sprouts were offered by Jangsuche Co., Ltd. (Korea). Nanopowdered peanut sprouts by the dry milling was supplied from Apexel Co. (Korea).

Particle size analysis

The particle size analysis was measured according to the procedure of Seo *et al.* (2009). Powdered peanut sprouts or nanopowdered peanut sprouts was mounted on a brass stub (10 mm in diameter) using two-sided adhesive tape. The stub surface was gently blown to remove unattached peanut sprout powders using a hand-held blower. The specimens were then made electrically conductive by coating under an argon atmosphere with a thin layer (approximately 30 nm in thickness) of platinum-palladium (8:2). The specimens were examined using a scanning electron microscope (SEM, Hitachi S-4700, Japan) operated at an accelerating voltage of 15 kV. The particle size of nanopowdered peanut sprouts was determined by Delsa (TM) Nano particle size analyzer (Beckman Coulter, USA).

Production of nanopowdered peanut sprouts-added Caciocavallo cheese

Caciocavallo cheese was produced using the method

described by Gobetti (2002). For the manufacture of nanopowdered peanut sprouts-added Caciocavallo cheese, 0.5% (w/v) of nanopowdered peanut sprouts was added into the raw milk. After pasteurization (LTLT) nanopowdered peanut sprouts-added milk was cooled down to 32°C. One percentage of a frozen concentrated starter culture (ABT-5, Chr. Hansen's Laboratory) was added to the milk. After adding 0.02% CaCl₂, the milk was set for 1 h and 0.025% rennet (double strength 290, Chr. Hansen's Laboratory) was added to the milk sample. Milk was then stirred quickly for 3 min and set to coagulate at 32 to 33°C for 45 min, and the curd was cut to size of 0.8×0.8×0.8 cm and the curd-whey mixture was gradually stirred during increasing to 48°C to cook for 30 min and pH reached to 5.8. After stirring, whey was drained as much as possible and the curd was relocated to one side of the vat. Matting the curds was done till reaching pH 5.2 and cut to 0.3 mm curd was stretched at 80°C. The stretched curds were shaped to a gourd dipper, 20% brine solution at 12°C for 1-2 h and ripened at 14°C for 6 wks at a relative humidity of 98%. Control and sample cheeses were made in duplicate in 2 batches.

Animals

Male DBA/1J mice (5 wks old; $n = 30$) were purchased from Central Lab. Animal Inc. (Korea). The mice were housed in plastic cages under the constant temperature of 25±3°C, and the constant humidity of 55±5% on 12 h light/dark cycle. Experimental diets were based on the AIN-76A diet (American Institute of Nutrition, 1977), as shown in Table 1. All the animal treatments and proce-

Table 1. Composition of AIN-76A Purified diet

Ingredient	%
Casein	20.0
DL-Methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fiver	5.0
Con oil	5.0
AIN Mineral mix ^a	3.5
AIN Vitamin mix ^b	1.0
Choline bitartrate	0.2

^aAIN-76 mineral mix (ing/kg): CaHPO₄, 500; NaCl, 74 potassium citrate monohydrate, 220; K₂SO₄, 52; MgO, 24; Mn carbohydrate, 3.5; Fe citrate, 6.0; Zn carbonate, 1.6; Cu carbonate, 0.3; KIO₃, 0.01; Na₂SeO₄·H₂O, 0.01; CrK(SO₄)·H₂O, 0.55; sucrose, 118.

^bAIN76 vitamin mix (in/kg): thiamine-HCl, 0.6; riboflavin, 0.6; pyridoxine-HCl, 0.7; nicotinic acid, 3; D-calcium pantothenate, 1.6; folic acid, 0.2; D-biotin, 0.02; cyanocobalamin, 0.001; retinylpalmitate, 0.8; DL- α -tocopheryl acetate, 20; cholecalciferol, 0.00025; menaquinone, 0.005.

dures were done according to the protocol of the institutional guidelines (The Institutional Animal Care and Use Committee, IACUC) at Sejong University (Korea).

Induction of collagen-induced arthritis and treatment

Induction of arthritis was followed by the procedure of Brand *et al.* (2007). Briefly, the mice were immunized intradermally on the base of the tail with 100 µm Bovine type II collagen (Sigma, USA) emulsified in Freund's complete adjuvant. On day 21, all mice were boosted with an intradermal injection [100 µm type II collagen emulsified in Freund's incomplete adjuvant (Gibco BRL, USA)]. After the induction of arthritis, the mice were divided into the following five groups depending on their diets: (1) normal, no immunization; (2) CIA, collagen-induced arthritis; (3) MTX, collagen-induced arthritis treated with methotrexate (0.3 mg/kg body weight); (4) CC, collagen-induced arthritis treated with Caciocavallo cheese (0.6 g/d); (5) NPCC, collagen-induced arthritis treated with nanopowdered peanut sprouts-added Caciocavallo cheese (0.6 g/d). During the 18 wks experimental periods, all mice were daily fed with 0.6 g of NPCC mixed in feed and allowed access to diets and distilled water *ad libitum*.

Measurement of clinical score and paw thickness

Clinical scores were measured according to the degree of inflammation: 0 = represented normal; grade 1 = swelling of one finger; grade 2 = swelling of more than two finger; grade 3 = swelling of heel; grade 4 = joint deformity with ankylosis (Brand *et al.*, 2007). The paw thickness in the hind paw was measured using a plethysmometer 7150 (Italy) from the initial injection day. Clinical scores and hind paw thickness were monitored every other wk for a period of 18 wks.

Biochemical analysis in serum

On the final day of the experiment, all of the mice were anesthetized with ethyl ether. The blood was collected from each by abdominal aorta and was placed into the serum separation tubes. After clotting at room temperature using serum separated tube, the serum was collected by centrifuging at 4000×g for 15 min. All serums were stored at -80°C until required. Cytokines [TNF-α (tumor necrosis factor α), IL-1β (interleukin-1β), and IL-6 (interleukin-1β)] and immunoglobulin G1 were measured by Luminex fluorescent bead mouse cytokine immuno assays (MILLIPLEX MAP, Millipore Corp., USA).

Histological assessment - H&E staining

After the mice were sacrificed, their right knee joints were collected and fixed in 10% natural buffered formalin. Tissues were decalcified in 10% ethylene diamine tetra acetate (EDTA) for 7 d and then embedded in a paraffin block. Tissue sections of 4 µm thickness were stained with hematoxylin and eosin (H&E staining). Photographs of the sections were taken using an Olympus IX70 (Japan) at the magnification of ×100.

According to the procedure of Mankin *et al.* (1971), the grade for histological changes was measured as follows: 0, normal; 1, surface irregularities; 2, pannus and surface irregularities; 3, clefts to transitional zone; 4, clefts to radial zone; 5, clefts to calcified zone; 6, complete disorganization.

Measurement of the spleen index

At the end of the experiment, all mice were sacrificed by decapitation. All the spleens of mice were weighed immediately after dissection. The spleen indexes were calculated by using the following formula as described previously (Chunxia *et al.*, 2011):

$$\text{Spleen index} = \frac{\frac{\text{spleen weight of CIA mice}}{\text{body weight of CIA mice}}}{\frac{\text{spleen weight of normal mice}}{\text{body weight of normal mice}}}$$

Statistical analysis

The results were expressed as mean±SD. Statistical significance was determined by analysis of variance and subsequent Duncan's multiple range test ($p < 0.05$). The analysis was performed using SAS statistical software.

Results and Discussion

Particle size analysis

The morphology of powdered peanut sprouts and nanopowdered peanut sprouts was observed by SEM, as shown in Fig. 1. The SEM images demonstrated that the particle size of powdered peanut sprouts apparently decreased during the manufacture of nanopowdered peanut sprouts. The average particle sizes of powdered peanut sprouts and nanopowdered peanut sprouts measured were about 50-150 µm (as measured by SEM) and about 300-350 nm in diameter (as measured by the particle size analyzer), respectively (Fig. 1 and 2).

Measurement of clinical score and hind paw thickness

The changes in the incidence and severity of arthritis in

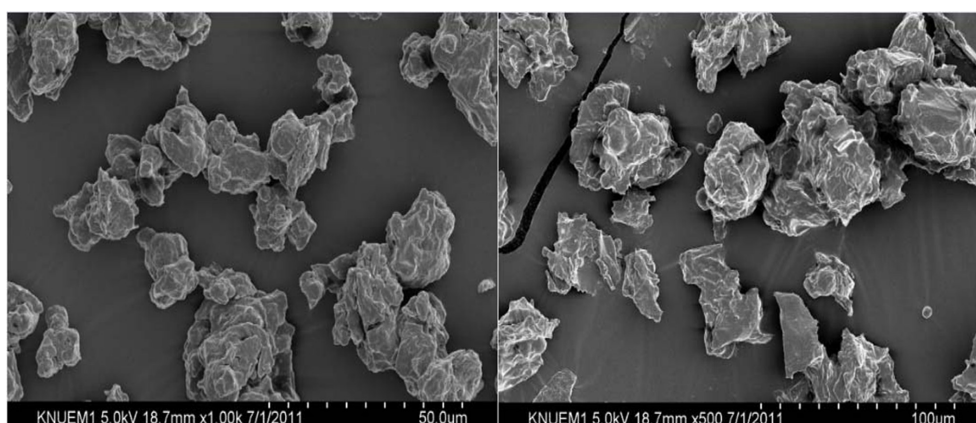


Fig. 1. Scanning electron microscope image for nanopowdered peanut sprout and powdered peanut sprouts.



Fig. 2. Particle size analysis of nanopowdered peanut sprouts.

CIA mice treated with nanopowdered peanut sprouts-added Caciocavallo cheese during 18 wks were shown in Fig. 3. For the all the groups, except for normal, the first sign of arthritis was observed at 2nd and 4th wks after immunization. The clinical arthritis scores for CIA, MTX, CC, and NPCC groups were dramatically increased until 6 wks and then the scores were not considerably changed. After 18 wks, the final clinical arthritis scores for CIA, MTX, CC, and NPCC were 3.39, 1.96, 2.79, and 2.67, respectively. This finding indicated that feeding nanopowdered peanut sprouts-added Caciocavallo cheese into the arthritic mice can reduce the incidence and severity of arthritis in mice with collagen-induced arthritis.

The values of the hind paw thickness for the MTX, CC, and NPCC groups were remarkably lower than that of the CIA group (Fig. 3). However, there was no considerable difference between CC and NPCC groups.

Level on TNF- α , IL-1 β , IL-6, and IgG1

In general, rheumatoid arthritis is a chronic, inflammatory, autoimmune disorder of the joints, for which current

treatment strategies remain suboptimal (Smolen and Steiner, 2004). Although the pathogenesis of the disease has not been fully understood, it contains cellular infiltration into synovial tissue and elevated inflammatory cytokines that lead to cartilage and bone erosion through the induction of matrix metallo proteinases (MMPs) and dysregulated chondrocyte/osteoclast function (Feldmann *et al.*, 1996; Goldring, 2003). Among the inflammatory cytokines, TNF- α seems to be on the top of a cytokine cascade, because it increases the levels of interleukin IL 1 β , and IL 6 (Goldring, 2003). According to Miani and Taylor (2000), TNF- α and IL-1 play major roles in the maintenance of chronic inflammation and/or tissue damage during the progression of rheumatoid arthritis. Furthermore, IL-6 is important inflammatory cytokine in rheumatoid arthritis, and it activates auto reactive T-cell, facilitates to produce rheumatoid arthritis factors, and derives to produce acute phase reactant, consequently affecting rheumatoid arthritis.

In the present study, the serum was collected from abdominal vein of DBA/IJ mice immunized with type II collagen. The TNF- α , IL-1, and IL-6 levels in the serum of the

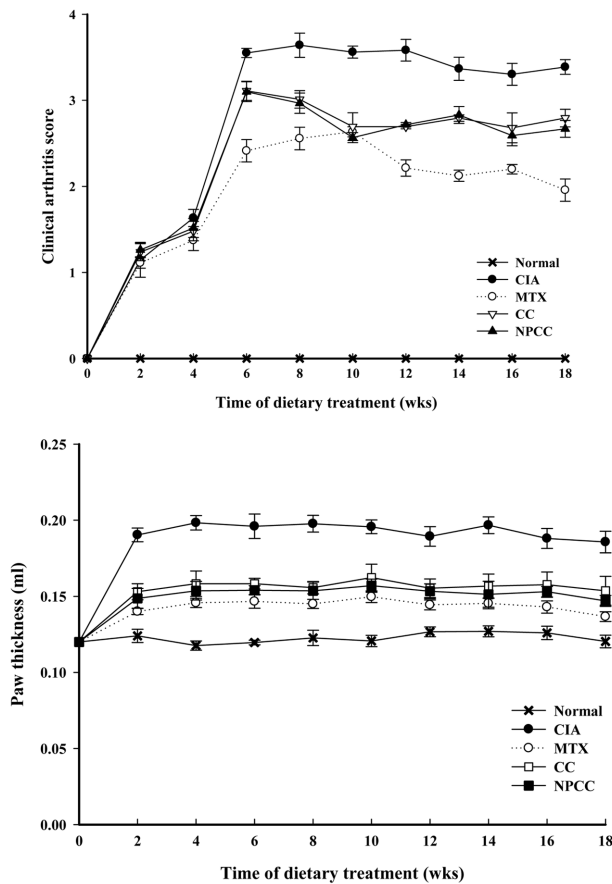


Fig. 3. Effects of Caciocavallo cheese or nanopowdered peanut sprouts-added Caciocavallo cheese on clinical scores of arthritis symptoms and hind paw thickness in arthritic mice: normal, no immunization; CIA, collagen-induced arthritis; MTX, collagen-induced arthritis treated with methotrexate; CC, collagen-induced arthritis powdered peanut sprouts treated with Caciocavallo cheese; NPCC, collagen-induced arthritis treated with nanopowdered peanut sprouts-added Caciocavallo cheese.

arthritic mice were analyzed by Luminex™ system and are presented in Fig. 4. The methotrexate is classified as an antimetabolite drug and is used to treating rheumatoid arthritis (Borchers *et al.*, 2004). Feeding methotrexate or nanopowdered peanut sprouts-added Caciocavallo cheese into the arthritis mice significantly decreased all of the TNF- α , IL-1 β , and IL-6 values as compared to the CIA group. Thus, the present study could offer the possibility of using nanopowdered peanut sprouts-added Caciocavallo cheese to reduce the symptom of rheumatoid arthritis.

The IgG1 levels in the serum of arthritis mice were presented in Fig. 5. The order for IgG1 levels in the arthritis mice groups was as follows: CIA > CC > NPCC > MTX. The IgG1 is one of most crucial rheumatoid arthritis factors and has been used for diagnosing rheumatoid arthritis. According to Xuzhu *et al.* (2013), IgG1 is mainly in-

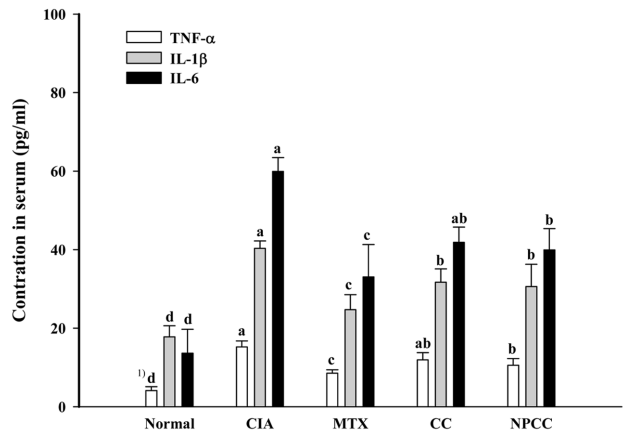


Fig. 4. Effect of Caciocavallo cheese or nanopowdered peanut sprouts-added Caciocavallo cheese on TNF- α , IL-1 β , and IL-6 in the serum of arthritis mice: normal, no immunization; CIA, collagen-induced arthritis; MTX, collagen-induced arthritis treated with methotrexate; CC, collagen-induced arthritis powdered peanut sprouts treated with Caciocavallo cheese; NPCC, collagen-induced arthritis treated with nanopowdered peanut sprouts-added Caciocavallo cheese. ¹⁾ Values with different superscripts are significant at $p < 0.05$ by Duncan's multiple range test.

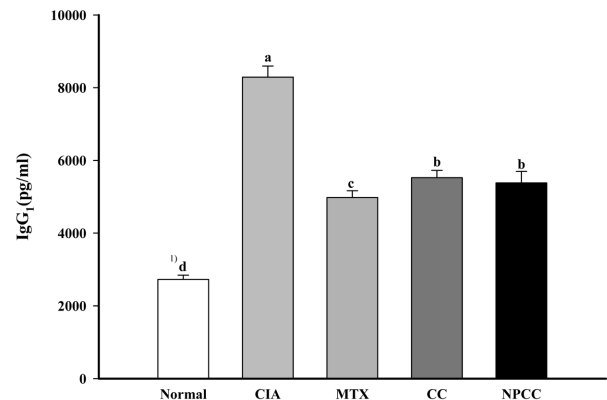


Fig. 5. Effect of Caciocavallo cheese or nanopowdered peanut sprouts-added Caciocavallo cheese on IgG1 in the serum of arthritis mice: normal, no immunization; CIA, collagen-induced arthritis; MTX, collagen-induced arthritis treated with methotrexate; CC, collagen-induced arthritis powdered peanut sprout treated with Caciocavallo cheese; NPCC, collagen-induced arthritis treated with nanopowdered peanut sprouts-added Caciocavallo cheese. ¹⁾ Values with different superscripts are significant at $p < 0.05$ by Duncan's multiple range test.

duced by type II cytokines (IL-4, IL-13). The decrease in the levels of IgG1 secreted from the body is a signal of decreased disease pathology (Viji *et al.*, 2010). Accordingly, the finding obtained from the present study on the reduction of the IgG1 level in the NPCC group may be an indication of attenuating rheumatoid arthritis in mice.

Histological analysis

Histological analysis was carried out by H&E staining for the hind knee joints of DBA/1J mice immunized with type II collagen (Fig. 6(A) and 6(B)). The normal group exhibited common articular cartilage, absence of infiltrate in the synovium, and open joints space. For the CIA group, the hind knee joints showed the typical and pathological changes with severe degrees of synovial inflammation and fibrosis, cartilage erosion, bone erosion, bone inflammation, fibrosis, and severe narrow joint space. On the other hand, the MTX and NPCC groups showed mild degrees of erosion, inflammation, and fibrosis in the hind knee joints of the mice, indicating the reduction of arthritis symptom in the mice.

Histological scores of the MTX and NPCC groups were significantly lower than that of the CIA group. Elmali *et al.* (2007) also reported that the dietary supplementation of resveratrol can suppress inflammatory arthritis in a rabbit model. This finding from Elmali *et al.* (2007) suggested that the significant decrease in the histological score for NPCC group in the present study could be related to the presence of resveratrol in nanopowdered peanut sprouts-added Caciocavallo cheese.

Spleen index

For measuring the spleen index of all the groups, the weights of spleen and body were recorded at the final day of experiment (Fig. 7). The spleen has central roles in the

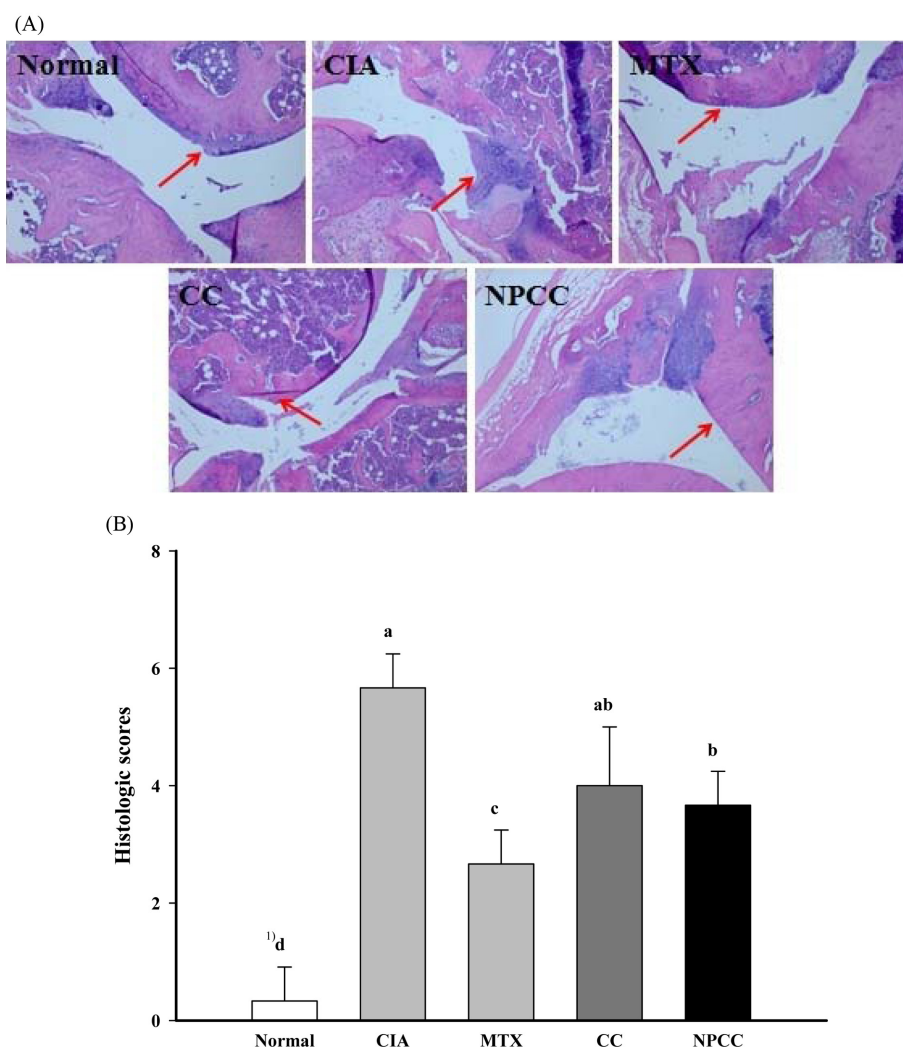


Fig. 6. Effect of Caciocavallo cheese or nanopowdered peanut sprouts-added Caciocavallo cheese on histological changes (A) and histological scores (B) in right knee joint of arthritic mice: normal, no immunization; CIA, collagen-induced arthritis; MTX, collagen-induced arthritis treated with methotrexate; CC, collagen-induced arthritis powdered peanut sprout treated with Caciocavallo cheese; NPCC, collagen-induced arthritis treated with nanopowdered peanut sprouts-added Caciocavallo cheese. The arrows in A showed the change of bone structure. ¹⁾ Values with different superscripts are significant at $p < 0.05$ by Duncan's multiple range test.

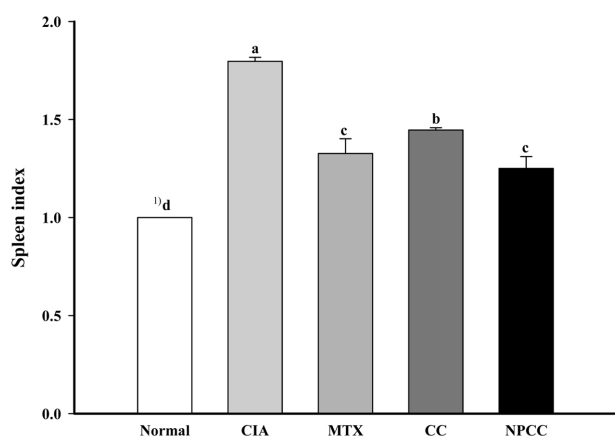


Fig. 7. Effect of Caciocavallo cheese or nanopowdered peanut sprouts-added Caciocavallo cheese on spleen indexes in arthritic mice: normal, no immunization; CIA, collagen-induced arthritis; MTX, collagen-induced arthritis treated with methotrexate; CC, collagen-induced arthritis powdered peanut sprouts treated with Caciocavallo cheese; NPCC, collagen-induced arthritis treated with nanopowdered peanut sprouts-added Caciocavallo cheese. ¹⁾ Values with different superscripts are significant at $p < 0.05$ by Duncan's multiple range test.

immune system. After the mice immunized with type II collagen, the weight of spleen was increased due to promoting immune system via production of antibodies and immunoglobulin (Hongfang and Meng, 2011). The spleen index for the CIA group was significantly higher than those for the MTX and NPCC groups. Interestingly, the dietary supplement of nanopowdered peanut sprouts-added Caciocavallo cheese had the similar effects on the reduction of spleen index in the arthritis mice compared with the methotrexate. Based on the finding, it was suggested that dietary supplementation (0.6 g/d) of nanopowdered peanut sprouts-added Caciocavallo cheese could inhibit the type II collagen-induced arthritis in mice.

Based on the results (clinical score, hind paw thickness, biochemical analysis, histological assessment, and spleen index) obtained from our study, there were no considerable differences between CC and NPCC models. In the present study, it was hypothesized that due to the advantage of size effect and high surface reactivity of nanoparticles, the addition of nanopowdered peanut sprouts would improve the reduction and treatment of arthritis symptom in the mice immunized with type II collagen. However, we did not find the more improved preventive effect of nanopowdered peanut sprouts-added Caciocavallo cheese on rheumatoid arthritis, as compared to Caciocavallo cheese. Accordingly, our study demonstrated that the addition of 0.5% (w/v) of nanopowdered peanut sprouts into Cacio-

cavallo cheese was not enough, so that the increase in the addition of nanopowdered peanut sprouts should be needed for the future study.

Conclusion

This study was designed to evaluate the influence of nanopowdered peanut sprouts-added Caciocavallo cheese on the prevention and treatment of rheumatoid arthritis in DBA/IJ mice immunized with type II collagen. Based on the findings (clinical score, paw thickness, serum analysis, and histological analysis), it is indicated that feeding nanopowdered peanut sprouts-added Caciocavallo cheese to the collagen-induced arthritis mice can reduce clinical score, paw thickness, TNF- α , IL-1 β , IL-6, IgG1, and histological scores in the mice. Therefore, the production of Caciocavallo cheese which incorporates nanopowdered peanut sprouts can broaden the utilization of peanut sprouts, and the products can be regarded as possible health-promoting nutraceutical foods, especially for the prevention and treatment of rheumatoid arthritis.

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