

Nucleoprotein Diet Ameliorates Arthritis Symptoms in Mice Transgenic for Human T-Cell Leukemia Virus Type I (HTLV-1)

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Summary Because rheumatoid arthritis (RA), an autoimmune disease, the patients often recognize side-effects due to the medication, alternative therapeutic strategies might potentially offer a clinical advantage. We evaluated the effect of nucleoprotein from salmon soft roe on animal model of arthritis. Mice transgenic for human T-cell leukemia virus type I (HTLV-1 Tg) were divided into three experimental groups and supplemented on either nucleoprotein-free (nonNP), or 0.6% or 1.2% nucleoprotein mixed (NP0.6 or NP1.2) diet for 3 months. The mice were evaluated arthritis by morphology, and measured with rheumatoid factor (RF). Moreover, macrophages and oxidative metabolites were assessed in the ankle and/or serum. Anti-oxidative potentials in nucleoprotein were determined with biological anti-oxidative potential (BAP) test, and electron spin resonance (ESR) analysis. NonNP-diet HTLV-1 Tg mice increased an arthritis symptoms and RF. The symptoms were ameliorated in NP-diet groups. Macrophages detected by F4/80 staining, and oxidative metabolites in the serum and/or joints were clearly decreased in 1.2% NP-diet HTLV-1 Tg mice. Nucleoprotein and DNA-nucleotide, but less protamine, had direct anti-oxidative potency with BAP test and/or ESR *in vitro*. These observations suggest that dietary nucleoprotein ameliorates arthritis symptoms in HTLV-1 Tg mice and offers hope as an alternative treatment for this debilitating medical condition.

Key Words: nucleoprotein, rheumatoid arthritis, alternative medicine, radical scavenger

Introduction

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disorder affecting ~1% of the world's population. RA is characterized by inflammation of synovial tissues in multiple joints, destruction of cartilage and bone, and joint deformation and enlargement [1]. The patients often

develop auto-antibodies and/or cellular immunity against various self-substances, including immunoglobulin G (IgG), also known as rheumatoid factors (RF) [2], type II collagen (IIC) [3, 4], and heat shock protein (HSP) [5, 6]. RF is accumulated in the joints and it is shown to enhance infiltration of inflammatory cells such as monocytes and lymphocytes. These in turn activate innate immune responses, resulting in elevation of pro-inflammatory cytokines and reactive oxygen species (ROS) in the synovial spaces and surrounding blood vessels. Synergistic responses are known to induce proliferation of the synovial membranes, causing arthritic deterioration resulting in a decrease in the joint functions [7–10].

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Clinical therapies for RA have been typically included medications such as disease-modifying anti-rheumatic drugs, anti-inflammatory agents, and immunosuppressants, and/or surgeries such as synovectomy. However, because the RA patients often recognize side-effects due to the medications, development of alternative approaches to be delayed the progression of the disease offers an attractive prospect.

Salmon is currently known as one of the most widely consumed fish in the world. According to the Japanese government, catch of salmon in 2006 was 219,000 metric tons, with the amount of soft roe associated with this catch thought to be an account for 5,000 metric tons. However, the fish soft roe is rarely used as food, largely because of difficulties associated with its processing. The soft roe usually serves only as a component of organic fertilizer and a feeds for domestic animals and farmed fish. The fish soft roe contains mostly nucleoprotein which mainly consists of a mixture of DNA-nucleotide (DNA-nu) (38%) and protamine (56%). Oral supplement of nucleotides has shown to influence the cellular metabolism, proliferation, differentiation and apoptosis, probably by acting as mediators of signal transduction [11–13]. Dietary nucleotides are also known to influence immune responses through type 1 and type 2 helper T (Th1 and Th2) cells [13–16], and DNA containing the base arrangement, TTAGGG has proven effective in the prevention or treatment of a variety of organ-specific and systemic autoimmune diseases, including arthritis in mouse models [17, 18]. A study has reported a possible anti-oxidative potential of nucleic acids [19]. Another main compartment, protamine is a protein which contains high levels of arginine [20]. Dietary protamine has been shown to prevent positively charged antigen amidated bovine serum albumin-induced arthritis due to its cationic properties [21].

Based on the line of evidences, it appears that nucleoprotein is an easily available natural product and may prove effective as an alternative medication for prevention or remedy of autoimmune diseases such as RA. However, to date, the nutritional significance, efficacy, and underlying mechanism of action on nucleoprotein remain poorly understood.

Previously, Iwakura *et al.* have reported the development of a transgenic (Tg) mouse carrying the *env-pX* region of HTLV-1, the etiologic agent of adult T-cell leukemia [22]. The histopathology observed in these Tg mice resembles human RA in some respects, such as showing the marked synovial and periarticular inflammations with articular erosion caused by invasion of granulation tissues. The HTLV-1 Tg mice also develop autoimmunity and show the signs of arthritis in multiple joints as early as 4 weeks of age. Depending on background, by 3 months of age 60% (BALB/c background) or 20% (C3H/HeN background) of the mice are affected [23].

In this study, we examined the dietary effect of nucleoprotein derived from salmon soft roe on progression of the arthritis in HTVL-1 Tg mice. Our results revealed that the pathological symptoms were ameliorated in the nucleoprotein additive chow diet HTVL-1 Tg mice, and suggested that anti-oxidative property in nucleoprotein might participate partially the amelioration of the arthritis as a putative mechanism.

Materials and Methods

Animals

HTLV-1 Tg mice, originally produced by injecting the LTR-*env-pX*-LTR region of the HTLV-1 genome into a (C3H/HeN × C57BL/6J) F1 ovum [22], were backcrossed to BALB/c mice (Sankyo Lab, Tokyo, Japan) for at least 10 generations before being used experimentally. These mice start to develop arthritis spontaneously at around 4 weeks of age, with 60% and 80% of the animals being affected at 3 and 6 months of age, respectively. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Showa University (#04116, 05092, 06092, and 07050). The mice were maintained under specific pathogen-free conditions in the animal facility of Showa University.

Experimental diets and design

The experimental chows were produced by Crea Japan (Tokyo, Japan). The chows based on an artificially formulated nucleoprotein-free chow (non NP) and that were supplemented with either 0.6% or 1.2% nucleoprotein in non NP (NP0.6 or NP1.2 chow, respectively) extracted from salmon soft roe (Table 1).

Male HTLV-1 Tg (total 84) and the wild-type (total 83) littermates were used for the study. The animals were divided into three experimental groups: non NP ($n = 43$ of HTLV-1 Tg and 34 of wild-type, respectively), NP0.6

Table 1. Composition of the experimental chow

% (w/w)	Experimental chow		
	Non-NP	0.6% NP	1.2% NP
Casein	24.5	24.5	24.5
Cornstarch	45.5	44.9	44.3
Granulated sugar	10	10	10
Corn oil	6	6	6
Crystal cellulose	3	3	3
Cellulose powder	2	2	2
α -Starch	1	1	1
Vitamin mixture	1	1	1
Mineral mixture	7	7	7
Nucleoprotein	—	0.6	1.2

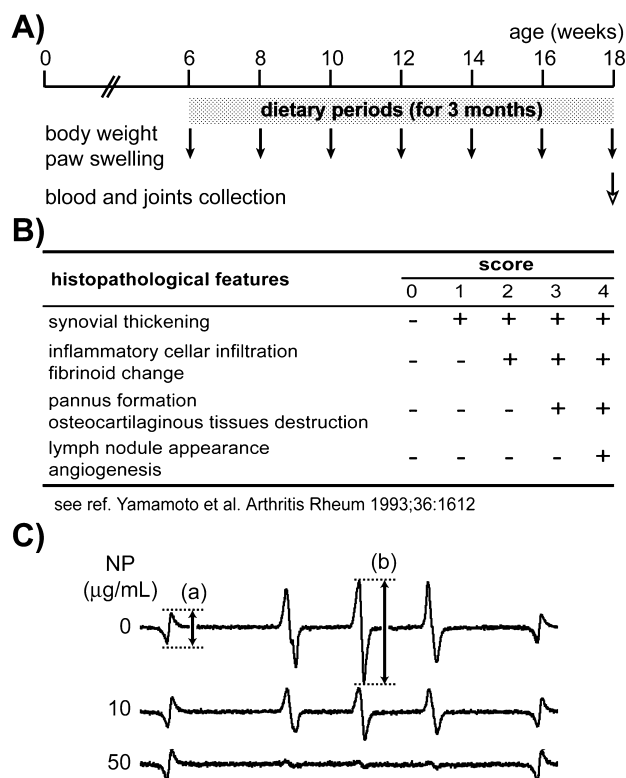


Fig. 1. Schematic diagram of the present study. (A) Diagram of animal study. Artificial experimental chows (see Table 1) are given for 3 months from 6 weeks of age. Body weight and paw swelling are measured every 2 weeks. Blood and histological analysis are carried out 18 weeks of age. (B) Histological criteria of arthritis according to previous paper [24]. (C) Representative images of wave pattern of $^1\text{O}_2$ in present/absent of nucleoprotein. The highest peak of $^1\text{O}_2$ generated by rose bengal is recorded without nucleoprotein (0 $\mu\text{g}/\text{mL}$, upper), but the height are decreased with nucleoprotein. (a) is peak of manganese which is internal control. (b) is peak of $^1\text{O}_2$. The relative $^1\text{O}_2$ generation was calculated by (b)/(a).

($n = 21$ and 23) or NP1.2 ($n = 20$ and 26). The experimental chows (non NP, NP0.6 or NP1.2) were given for 3 months from 6 to 18 weeks of age. During the periods, the animals were weighed and measured paw thickness every 2 weeks. At the end of the 3 months, they were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and in some cases blood samples were collected from the right ventricle for measurement of RF and oxidative metabolites. All animals were subsequently perfused transcardially with saline, followed by 10% buffered formalin for preparing paraffin sections or by 2% paraformaldehyde for preparing frozen sections (Fig. 1A).

Measurement of paw swelling

The thicknesses of the wrist and ankle joints were measured with electron micrometer calipers (QUICK mini PK-1012; Mitutoyo, Kanagawa, Japan). The long and short axes of the joints were each measured 3 times, and the average was calculated. The mean thickness of the joints was then corrected by body weight, and experimental paw swelling score was calculated as a percentage of joint thickness at 6 weeks of age. Score 0 was 100% or less, Score 1 was from 100% to less than 110%, Score 2 was from 110% to less than 120%, and Score 3 was 120% or more. HTLV-1 Tg ($n = 39$, 18 and 20 for the non NP, NP0.6 and NP1.2 diet groups, respectively) and wild-type ($n = 10$, 8 and 18, respectively) mice were assessed.

Enzyme immunoassay

Serum obtained from 18 weeks of age was determined the level of RF using mouse IgG Rheumatoid Factor ELISA KIT (AKRRG-101, Shibayagi, Gunma, Japan), according to the manufacturer's instructions. The data are presented as units (U)/ml, with one unit representing the titer against 1 mg/ml mouse IgG.

Morphological evaluation

The limbs ($n = 10$ – 16) were trimmed the skin and muscles, decalcified in 1.0% formic acid for 7 days and paraffin-embedded. The limbs were cut into 4- μm -thickness sections in the longitudinal axis for histological examination. The sections were stained with hematoxylin-eosin (HE) and toluidine blue (TB) and histopathologically evaluated graded on a scale of 0–4 in the wrist and ankle joints as previously described [24]. Grade 0 was representing no observed abnormalities. Grade 1 changes were characterized by proliferation of synovial lining cells, grade 2 by infiltration of inflammatory cells, grade 3 by the destruction of cartilage and bone, and grade 4 by the formation of lymphoid follicles and vascular changes (Fig. 1B). An investigator (S.Y) who was unaware of the dietary group to which each animal belonged determined the morphological scores.

Immunohistochemistry

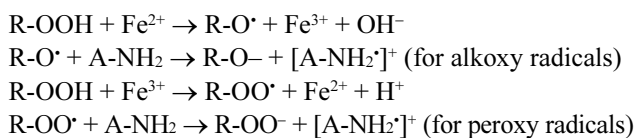
The fixed limbs ($n = 3$ – 4 mice per group) were decalcified in 14% EDTA for 7 days at 4°C . After washing with 0.1 M phosphate buffer (PB; pH 7.2), the limbs were immersed in 0.1 M PB containing 20% sucrose for 2 days, and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). The sections (8 μm -thickness) were applied for immunostaining. After treatment H_2O_2 and 5% normal horse serum, the sections incubated with polyvalent goat anti-mouse IgG antibody (1:320,000; Sigma, St Louis, MO), rat anti-F4/80 antibody (1:500; BMA, Augst, Switzerland), or rabbit anti-3-nitrotyrosine (3-NT) antibody (1:500, Upstate Biotechnology, Lake Placid, NY). The sections were

then rinsed and incubated with biotinylated horse anti-goat IgG (1:200, Vector, Burlingame, CA), rabbit anti-rat IgG (1:200, Vector), or goat anti-rabbit IgG (1:200, Santa Cruz) for 2 h, respectively. The reaction was visualized using an avidin/biotin complex (Vector) with diaminobenzidine (DAB; Vector) as a chromogen. Controls followed the same protocol except that tissues were incubated without the primary antibodies.

Determination of oxidative metabolite

An oxidative metabolite as a marker for oxidative stress in serum was determined by a commercial kit, colorimetric determination of reactive oxygen metabolites test (d-ROMs test) using free radical electron evaluator (FREE, Health & Diagnostics, Naples, Italy). The number of samples in non NP-, NP0.6-, and NP1.2 diet HTLV-1 Tg mice were 19, 11 and 16, respectively.

Briefly, serum samples (20 μ l) were dissolved at 37°C in acetate buffer (pH 4.8) containing FeCl₂. The solution was then mixed gently, and 20 μ l of chromogenic mixture containing an aromatic alkyl-amine were added. After incubation for 5 min at 37°C, the production of the pink aromatic derivative was measured at 546 nm according to the reaction as follows:

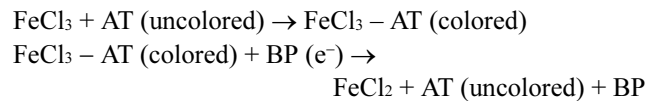


R-OOH; hydroxyperoxides, R-O \cdot ; alkoxy radicals, OH \cdot ; hydroxyradical, A-NH₂; aromatic amine, [A-NH₂]⁺; pink-colored aromatic derivative, R-OO \cdot , peroxy radicals.

The results were expressed as unit (U). One unit coincided with the oxidative potentials of 0.08 mg H₂O₂/dl.

Assay of biological antioxidant potential test (BAP test) of NP *in vitro*

An antioxidant potential of nucleoprotein was measured as a commercial kit, BAP test, using FREE according to instruction manual with minor modification. In brief, nucleoprotein, or DNA-nucleotide or protamine both of which is a component of nucleoprotein was mixed well with distilled water. The aliquots (10 μ l) were mixed with reactive solution and the absorbance determined at 510 nm immediately prior to initiation of the reaction. The mixture was then incubated for 5 min at 37°C. After centrifuging at 4,000 \times g for 2 min to remove insoluble matter, the post-reaction absorbance of the mixture was measured at 510 nm. Under these conditions, the solution loses color, the intensity of this chromatic change being directly proportional to the ability of the incubated sample to reduce ferric ions to ferrous ions (μ mol/liter) as follows:



FeCl₃; ferric chloride, AT (uncolored); a thiocyanate derivative (uncolored), FeCl₂ – AT (colored); the colored complex of ferric chloride with the thiocyanate derivative, BP (e⁻); a sample barrier with reducing/electron donating/antioxidant activity against ferric ions, BP; the oxidized form of BP (e⁻), FeCl₂; the ferrous chloride obtained by the reducing activity of BP (e⁻).

ESR analysis of NP

To identify sort of ROS scavenged by nucleoprotein, we carried out ESR analysis. Singlet oxygen (¹O₂) was generated by a photosensitization reaction with rose bengal (Wako, Tokyo, Japan). ¹O₂ was indirectly estimated as the peak intensity of 2,2,6,6-tetramethyl-4-hydroxy-piperidinyloxy (4-OH TEMPO) radical produced by the oxidation of 2,2,6,6-tetramethyl-4-hydroxy-piperidine (4-OH TEMP) [25, 26] with ¹O₂ (produced by photosensitization with rose bengal), using ESR (JEOL JES, X-band, 100 kHz modulation frequency, JEOL Corporation, Tokyo Japan) [27]. NP was dissolved in dimethylsulfoxide (DMSO) to the final concentrations of 5, 10, 20 and 50 μ g/ml (*n* = 3). Samples, phosphate buffer (pH 7.4, 80 μ l), 100 mM 4-OH TEMP (40 μ l) and 200 μ M rose bengal (20 μ l), and each sample (in 60 μ l DMSO) transferred to quartz ESR cell (Radical research Company, Tokyo Japan) for recording the ESR spectrum. The instrument settings were as follows: center field, 335.5 \pm 5 mT; microwave power 5 mW; modulation amplitude, 0.1 mT; gain, 2.5 \times 100. ¹O₂ generation was calculated by following formula (Fig. 1C).

$$^1\text{O}_2 \text{ generation} = (\text{b})/(\text{a}).$$

(a): peak height (intensity) of manganese as an internal control.

(b): peak height (intensity) of ¹O₂ in each sample.

No nucleoprotein which means DMSO only was assigned as 100%. Radical scavenging activity was determined by decreasing of ¹O₂ generation.

Statistical analysis

Data were expressed as mean \pm SEM. One-way ANOVA was performed, followed by Dunnett's *post hoc* test for comparison with vehicle-treated HTLV-1 Tg or wild-type mice. Values of *p* < 0.05 were considered statistically significant.

Results

Clinical finding

Artificially formulated experimental chows, nonNP, NP0.6 and NP1.2 were fed to HTLV-1 Tg and wild-type mice for 3 months from 6 to 18 weeks of age. At the end of this period, the survival rate in the nonNP-, NP0.6- and NP1.2-diet HTLV-1 Tg groups were 69.8 (30 of 43 mice), 81.0 (17 of 21 mice) and 85.0 (17 of 20 mice) %, respectively, these differences being insignificant by Kaplan-Meier survival analysis. The corresponding survival rates in the nonNP, NP0.6 and NP1.2 groups in wild-type mice were 91.2 (31 of 34 mice), 100.0 (23 of 23 mice), and 96.2 (25 of 26 mice) %, respectively.

As shown in Fig. 2A, at 6 weeks of age corresponding before diet, the mean body weight of the HTLV-1 Tg mice (22.4 ± 0.3 g; mean \pm SEM) was already less than that of the wild-type mice (25.4 ± 0.4 g). The body weight of wild-type mice gradually increased between 32 and 34 g at 18 weeks of age (no significantly difference in three experimental groups). The addition of NP could be no influence total calorie gain or consumption. By contrast, at 18 weeks of age, the mean body weight of the HTVL-1 Tg mice was 23.8 ± 0.9 g, 25.8 ± 1.3 g, and 26.8 ± 0.7 g in the non NP, NP0.6 and NP1.2 diet groups, respectively. NP1.2-fed Tg mice were significantly greater body weight than non NP-fed one at both 16 ($p < 0.01$) and 18 ($p < 0.05$) weeks of age.

Effect of NP diet on paw swelling

Joint thickness was measured in the different groups of HTLV-1 Tg and wild-type mice from 6 to 18 weeks of age (Fig. 2B and C). In the wild-type littermates, each dietary cohort consistently recorded a paw swelling score of 0.25 or less in both the wrists and ankles, with no significant differences. In the case of the non NP-diet HTLV-1 Tg mice, the score gradually increased, and showed maximum at 18 weeks in the wrist (1.13 ± 0.26) and at 16 weeks in the ankle (1.23 ± 0.24). Each joint of NP-fed groups in Tg mice was lower scores than that of the non NP-fed Tg mice, with the wrists and ankles in the NP1.2-fed Tg group being 0.33 ± 0.18 and 0.44 ± 0.18 ($p < 0.05$) at 16 weeks, and 0.25 ± 0.14 ($p < 0.01$) and 0.13 ± 0.09 ($p < 0.001$) at 18 weeks, respectively.

Improvement of histopathological score with nucleoprotein

Histopathological changes in animals with arthritis were next evaluated at 18 weeks of age. In Fig. 3A, Representative images of ankles were shown in wild-type, and non NP or NP-diet HTLV-1 Tg mice. The ankles of the non NP-fed HTLV-1 Tg mice showed marked proliferation of synovial tissues, increases of inflammatory cells, destruction of cartilage and excessive deformation of bone. Moreover, the formation of lymphoid follicles and angiogenesis in the

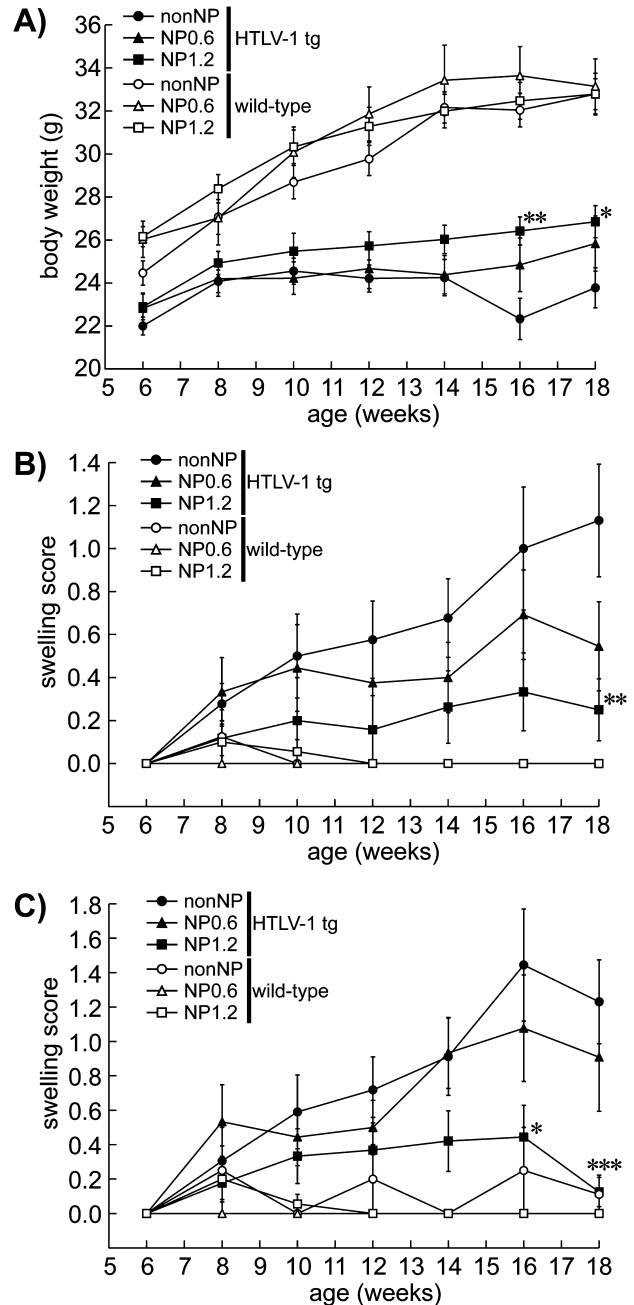


Fig. 2. Changes in body weight and joint swelling in HTLV-1 Tg and wild-type mice. The body weight (A), and thickness of the wrists (B) and ankles (C) are measured for 3 months from 6 to 18 weeks of age. Joint thickness was showed as the paw swelling score (see Materials and Methods). NonNP-fed HTLV-1 Tg mice tend to have low body weights and greater paw swelling scores during the experimental period. NP1.2-fed HTLV-1 Tg mice recorded a significantly improved body weight and paw swelling score at 16 and/or 18 weeks of age. Each value is expressed as mean \pm SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with nonNP-fed HTLV-1 Tg or wild-type mice (Dunnett's *post hoc* test).

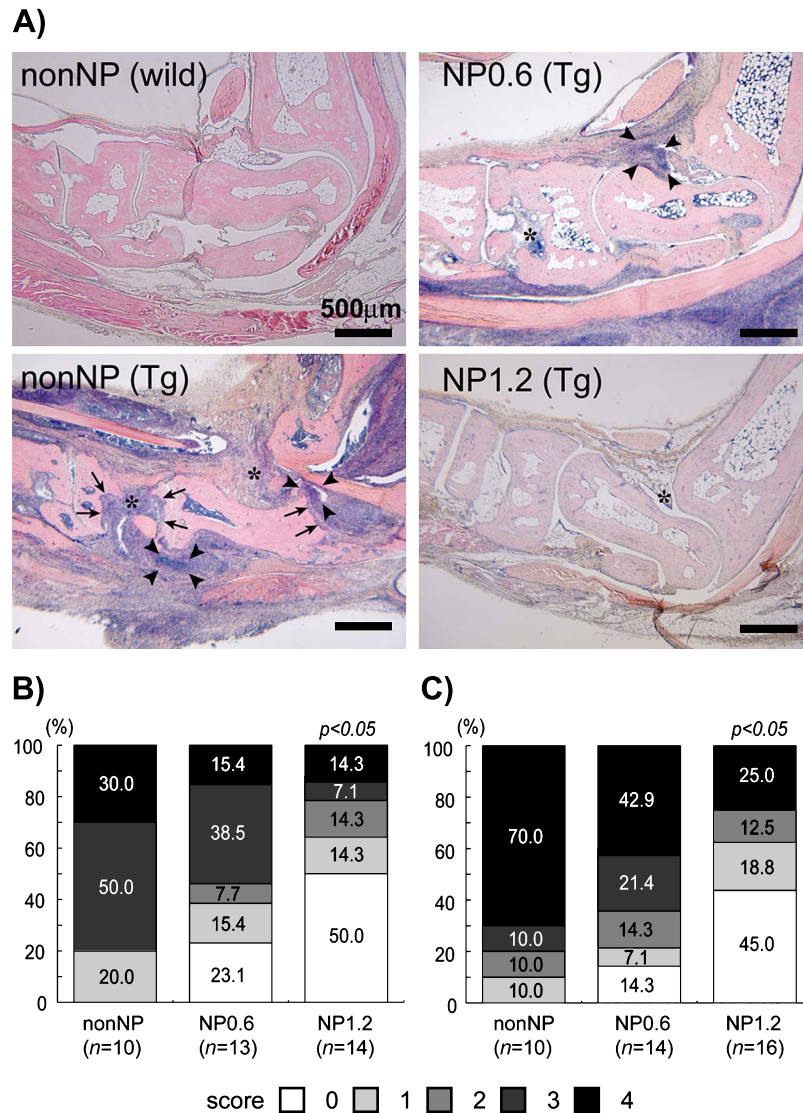


Fig. 3. Histopathological score of arthritic degeneration in the wrists and ankles after NP diet. (A) Typical histopathological images (HE staining) of the ankles in the nonNP-fed wild-type (wild), and nonNP-, NP0.6- or NP1.2-fed HTLV-1 Tg (Tg) mice. Ankle in nonNP-fed Tg mice clearly showed proliferation of synovial tissue (asterisks), inflammation and lymph nodes (arrowheads), and destruction of cartilage and bone (arrows). Eighty percentages of nonNP-fed HTLV-1 Tg mice recorded a score of 3 or 4 in wrists (B) and ankles (C). The mice fed nucleoprotein additive chow are ameliorated the score in a content-dependent manner. NP1.2-fed HTLV-1 Tg mice had a significantly reduce the score compared with nonNP-fed animals ($p < 0.05$). Numbers in the columns indicate the incidence (%).

proliferation of synovial tissues were also apparent at higher magnification. The arthritis in NP-fed groups of Tg mice were clearly improved.

The histopathological scores in the wild-type mice were mostly 0, only rarely rising as high as 1 (data not shown). Then, the histopathological features (Fig. 1B) were scored from 0 (normal) to 4 (severe) in the wrists (Fig. 3B) and ankles (Fig. 3C) of HTLV-1 Tg mice as described in a previous study [24]. Eighty percentages of the non NP-fed Tg mice recorded a score of 3 or more in their wrists and ankles at 18 weeks of age. In NP0.6-fed Tg animals, the

score of 3 or more was recorded in 53.9% and 74.3% of cases in the wrists and ankles, respectively, with synovial tissue proliferation and inflammatory cells also being noted (Fig. 3A). In the case of NP1.2-diet Tg mice, the histopathological score was significantly decreased to compare with the non NP-fed animals, with a score of 3 or more being recorded in 21.4% ($p < 0.05$) and 25.0% ($p < 0.05$) of wrists and ankles, respectively. The histopathological scores in the wild-type mice were mostly 0, only rarely rising as high as 1 (data not shown).

Decreases of serum and tissue IgG (RF)

Serum RF was measured in HTLV-1 Tg and the wild-type mice at 18 weeks of age (Fig. 4A). There were no significant differences the serum RF levels in the non NP-, NP0.6- and NP1.2-fed wild-type animals. In contrast, the level in the non NP-fed HTLV-1 Tg was 22.8 ± 2.1 U/ml ($n = 11$), approximately 1.5-fold higher than that in the wild-type mice. In NP0.6- and NP1.2-fed HTLV-1 Tg mice, the value decreased to 19.4 ± 2.4 ($n = 12$) and 16.1 ± 1.5 U/ml ($n = 15$, $p < 0.05$ vs non NP-fed HTLV-1 Tg one), respectively.

The incremental changes in serum RF levels were verified by mouse IgG immunostaining in the joints (Fig. 4B). Few IgG-positive reactions were observed in the ankles of non NP-fed wild-type animals. However, intense and widespread immunoreactivity was noted in the ankles of the non NP-fed HTLV-1 Tg animals. The observed IgG-positive reactions were most obvious around the bones, especially in the synovial tissues, but were less apparent in the muscles. The IgG-like immunoreactivity was decreased in NP0.6- and NP1.2-fed animals, with the ankle joints of NP1.2-fed HTLV-1 Tg mice appearing similar to those of the wild-type mice. These findings were mirrored by those obtained in the wrist joints (data not shown).

Decreases in the monocytes infiltration with NP

Immunostaining for F4/80 which is a marker for macrophage was carried out in order to further investigate the monocytes infiltration in joints as an inflammatory response (Fig. 5). F4/80 immunoreactivity was observed in the bone cavity of the non NP wild-type mice due to the presence of hematopoietic cells in the bone marrow, but less labeled in the synovial cavity and surrounding area in the ankle joint. The number and intensity for F4/80 immunopositive cells were prominent in these regions in the non NP-fed HTLV-1 Tg mice (Fig. 5A). Higher magnification images revealed small round and/or amorphous shaped F4/80-positive cells clustered in the synovial membrane. F4/80 immunoreactivity was also increased on the surface of the deformed bone. In mice with the supplement of nucleoprotein, the F4/80-positive responses were clearly reduced in a nucleoprotein dose-dependent manner (Fig. 5B and C).

We measured the serum levels of IL-6 and IL-17 at 18 weeks of age to further investigate inflammatory responses. IL-6 and IL-17 levels increased in HTLV-1 Tg mice compared with their wild-type counterparts. However, no significant differences were recorded as a result of supplement of nucleoprotein in HTLV-1 Tg one. (data not shown).

Suppression of oxidative stress by NP

It is supposed that the oxidative stress participated in the arthritis. Therefore we measured a serum oxidative metabolite with dROMs test, and a tissue oxidative metabolite, 3-

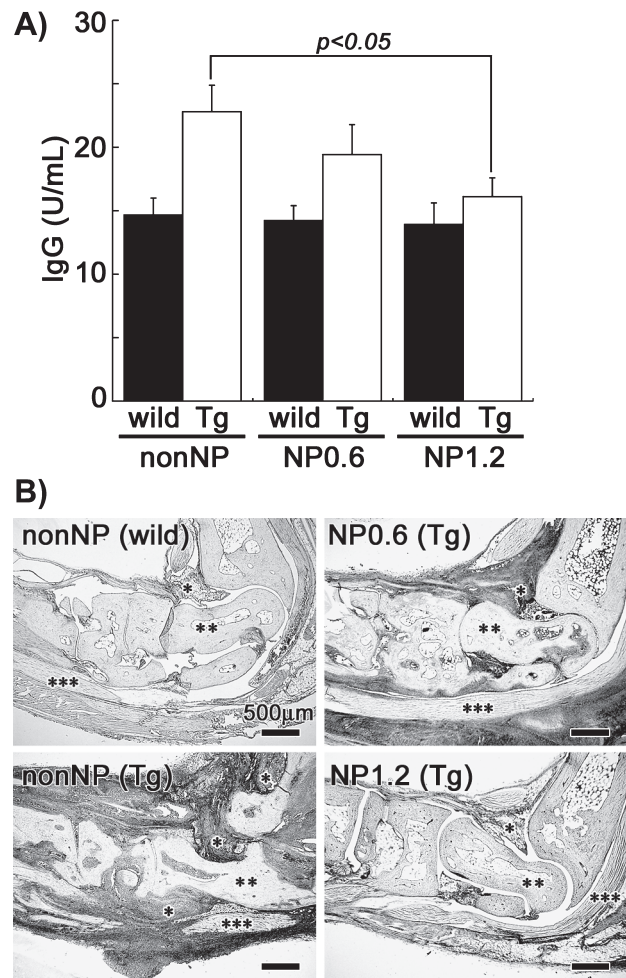


Fig. 4. Dietary effect of nucleoprotein on RF (as IgG) in HTLV-1 Tg (Tg) and wild-type (wild) mice. (A) RF level in serum of nonNP-fed HTLV-1 Tg mice was higher than that of the wild-type mice. NP-fed groups displayed a dose-dependent decrease in serum RF, with NP1.2-fed HTLV-1 Tg mice recording a significant decrease in the level of RF compared with that in the nonNP-fed group ($p < 0.05$). Each value is shown as mean \pm SE. (B) Immunohistochemical staining of mouse IgG in the ankles of nonNP-fed wild-type (wild), and nonNP-, NP0.6- or NP1.2-fed HTLV-1 Tg (Tg) mice. Little reactivity was seen in the ankles of wild-type mice. By comparison, intensity of IgG immunoreaction in the ankles was markedly stronger in nonNP-fed HTLV-1 Tg mice, but was decreased in NP0.6- or NP1.2-fed groups. The IgG-positive reactions in the nonNP-fed HTLV-1 Tg mice were observed in the synovial tissue (*) and, to a lesser extent, in the bone (**) and muscles (***).

NT. dROMs test represents the metabolites for peroxy- and alkoxy-radicals, whereas 3-NT is the oxidative metabolite of L-tyrosine by peroxynitrite which is reactant between superoxide anion and nitric oxide. As shown in Fig. 6A, serum dROMs value in the non NP-fed HTLV-1 Tg mice

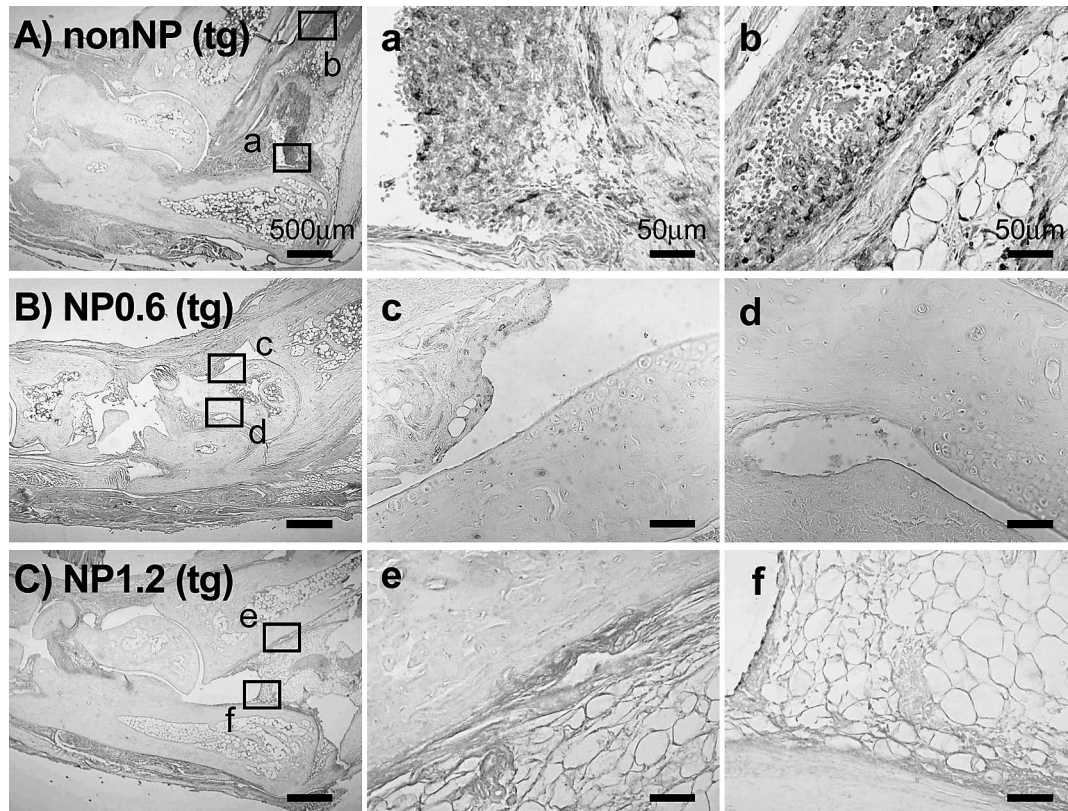


Fig. 5. Dietary effects of nucleoprotein on inflammatory responses in HTLV-1 Tg mice. Immunohistochemical staining for a macrophage marker, F4/80, in the ankle of nonNP (A)-, NP0.6 (B)- or NP1.2 (C)-fed HTLV-1 Tg mice. A higher magnification image of the boxed regions is shown in (a) to (f). F4/80 immunoreactivity was clearly observed in the ankles of nonNP-fed HTLV-1 Tg mice, especially in the synovial membrane (arrows in *a* and *b*). The number of labeled cells and the intensity of F4/80 immunoreactivity decreased in a dose-dependent manner. F4/80 immunoreactivity was reduced or absent in the synovial membrane of the NP1.2-fed HTLV-1 Tg mice.

was 159.9 ± 9.9 U ($n = 19$). This level decreased dose-dependently in the NP0.6- and NP1.2-fed HTLV-1 Tg animals to 139.2 ± 10.4 U ($n = 11$) and 125.2 ± 7.1 U ($n = 16$, $p < 0.05$ vs non NP Tg). Immunostaining for 3-NT in the ankles verified the increase in oxidative stress in the non NP-fed HTLV-1 Tg mice (Fig. 6B) and the positive reaction was clearly located in the hyaline cartilage. However, no such immunoreactivity was observed in the NP1.2-fed HTLV-1 Tg and non NP-fed wild-type mice.

Anti-oxidant potential of NP in vitro

Finally, we determined anti-oxidative potential of nucleoprotein *in vitro* whether the oxidative stress was scavenged by nucleoprotein or not. In addition, to determine what component is required for the radical scavenging, we carried out the BAP test against the main component of nucleoprotein, DNA-nu and protamine as well as nucleoprotein (Fig. 7A). A nucleoprotein suspension which includes approximately 38% DNA-nu and 56% protamine displayed direct radical scavenging ability in a dose-dependent manner. The BAP value of nucleoprotein was significantly

increased at $5.0 \mu\text{g/ml}$ or more. DNA-nu was higher BAP value than nucleoprotein, and showed significantly increase at $2.5 \mu\text{g/ml}$ or more, probably for high DNA-nu content. Protamine also exerted significantly anti-oxidative capability at $75 \mu\text{g/ml}$ or more while it was obviously less than the others. DNA-nu looks higher radical scavenging potential. However, correcting the content of DNA-nu in nucleoprotein, both samples show similar radical scavenging ability. This also indicates the radical scavenging ability might be due to DNA-nu.

Further analysis by ESR demonstrated that nucleoprotein could scavenge artificial generated $^1\text{O}_2$ in a dose-dependent fashion (Fig. 1C and 7B). Nucleoprotein decreased $^1\text{O}_2$ to 61.20 ± 0.63 , 50.14 ± 0.64 , 25.17 ± 1.43 , and $3.30 \pm 0.20\%$ at 5, 10, 20, and 50 $\mu\text{g/ml}$, respectively. Nucleoprotein also could scavenge slightly NO radical and superoxide anion while the higher NP concentrations (milligrams order) were required for the significantly difference (data not shown).

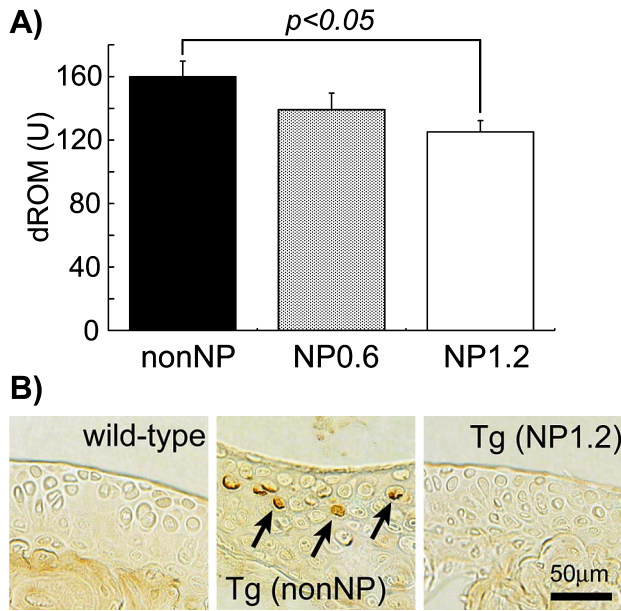


Fig. 6. Dietary effect of nucleoprotein on oxidative stress in HTLV-1 Tg mice. (A) the mice fed nucleoprotein additive chows decreased the serum level of oxidative metabolite measured with ROM test, being significantly lower in NP1.2-fed HTLV-1 Tg mice than in nonNP-fed group ($p < 0.05$, Dunnett's *post hoc* test). Each value is shown as mean \pm SEM. (B) Immunohistochemical staining of 3-NT in hyaline cartilage of the ankles in nonNP-fed wild-type, and nonNP- or NP1.2-fed HTLV-1 Tg (Tg) mice. There was marked 3-NT immunoreactivity in the nonNP-fed HTLV-1 Tg group (arrows). However, few positive reactions were observed in the nonNP-fed wild-type or NP1.2-fed HTLV-1 Tg mice.

Discussion

We have demonstrated in the present study that the artificial chow supplement with nucleoprotein derived from salmon soft roe suppresses 1) a decline in body weight, 2) joint hypertrophy, 3) an increases in RF level, and 4) aggravation of arthritis in HTLV-1 Tg mice in a dose-dependent manner. We have also determined that Tg mice given nucleoprotein additive chow decreased macrophages activation or monocyte infiltration in the joints, and oxidative metabolites. The latter effects may appear to result from the direct radical scavenging ability of nucleoprotein, especially of DNA-nu.

Iwakura *et al.* have previously reported that 60% and 80% of HTLV-1 Tg mice develop RA-like symptoms at 3 and 6 months of age, respectively [22, 23]. In the present study, the incidence of arthritis in the non NP-fed HTLV-1 Tg animals had already accounted for 80% at 18 weeks (4.5 months) of age. This slightly exacerbation of the course of these symptoms is possibly due to the lack of any nucleoprotein in

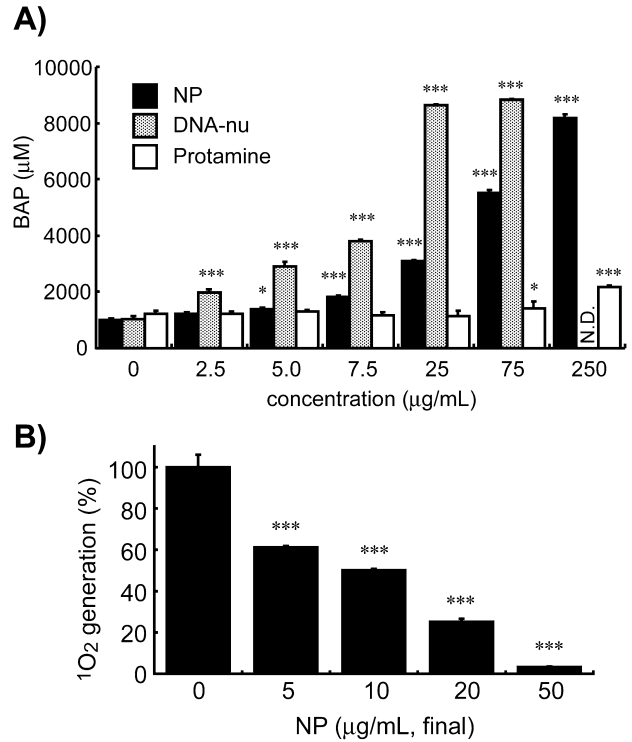


Fig. 7. Nucleoprotein and DNA-nu, but less protamine exert direct anti-oxidant capacity. (A) Nucleoprotein, DNA-nu, and protamine suspensions were performed BAP test with FREE. Nucleoprotein (close bar) and DNA-nu (gray bar) increase significantly at 5.0 and 2.5 $\mu\text{g}/\text{ml}$ or more, respectively, and show strong anti-oxidative potential. In contrast, protamine suspension (open bar) also exerts anti-oxidative potential (75 $\mu\text{g}/\text{ml}$ or more), but is obviously less than the others. Each data is shown Mean \pm SEM ($n = 5$). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ to compare with 0 $\mu\text{g}/\text{ml}$ of each group (Dunnett's *post hoc* test). N.D: no data. (B) Quantitative analysis of $^1\text{O}_2$ with several concentration of nucleoprotein. The height of peak on the $^1\text{O}_2$ is decreased in a dose-dependent manner. Each data is shown Mean \pm SEM ($n = 2 - 3$). ***: $p < 0.001$ to compare with 0 $\mu\text{g}/\text{ml}$ of each group (Dunnett's *post hoc* test).

the experimental chow. HTLV-1 Tg mice fed nucleoprotein additive chow significantly improved the clinical manifestations of the arthritis in a dose-dependent manner. Apparently, there are no differences in any experimental chow fed wild-type mice. Privation of nucleoprotein in daily diet might influence easily in a pathological condition while privation of it in healthy condition might be less influence.

The main components of nucleoprotein from salmon soft roe are DNA-nu and protamine. To date, dietary nucleic acids and nucleotides have been shown to influence optimal growth, cell functions and the immune responses [13, 28]. Nucleic acids reduce oxidation of linoleic acid *in vitro* [19], whereas nucleotide-free diets result in a decrease in cellular

and humoral immune responses [14, 28, 29] as well as reduced resistance to bacterial and fungal pathogens [30–32]. According to the previous reports, we have initially postulated modification of the immune responses such as reduction of pro-inflammatory cytokines. However, in present study, no significant differences were observed on proinflammatory cytokine levels among the experimental chow diet although macrophages activation or infiltration in the joints was clearly decreased. Proinflammatory cytokines bring about innate activation of macrophages, synovial tissues and monocytes [22, 33, 34]. The innate activated inflammatory cells could produce ROS [35–37]. Increases in oxidative stress have been implicated in the progression of symptoms in RA sufferers. The anti-RA medications, Methotrexate and Etanercept reduce oxidative stress and are associated with an improvement in RA symptoms [38–40]. We therefore shifted our focus to anti-oxidative potential of nucleoprotein because one previous paper has been reported to nucleic acids suppress oxidative stress [19]. Our findings clearly revealed that NP1.2-fed HTLV-1 Tg mice was significantly less in serum and joint oxidative metabolites levels compared with non NP-fed HTLV-1 Tg mice. Moreover, *in vitro* study indicated that nucleoprotein, especially the component DNA-nu had a free radical scavenging effect, but protamine was less. Indeed, nucleoprotein could scavenge mainly $^1\text{O}_2$ by ESR analysis. These results suggest that a preventive mode of action of dietary nucleoprotein suppresses the oxidative stress and might result in the prevention of infiltration and/or activation of inflammatory cells in part. In contrast, there have been reports that protamine or basic polypeptides such as poly-L-arginine and poly-L-lysine participate in suppression of arthritis due to its cationic properties [21]. Protamine or basic polypeptides also inhibit the activity of synovial phospholipase A2 patients with RA [41]. Endothelial cells in the luxuriant capillaries in the synovial tissues from patients with RA stimulated synovial cell proliferation. The protamine inhibited the synovial cell proliferation [42]. These studies lead to support to the idea that protamine may also act additively or synergistically with DNA-nu to prevent the progression of arthritis as well as DNA-nu. We need further to study by using separated substances of nucleoprotein what component of nucleoprotein is responsible for the suppression of the arthritis and how much the suppression of oxidative stress contributed to the amelioration of arthritis.

In conclusion, we have provided an excellent use for nucleoprotein obtained from fish soft roe, which at present is a huge untapped natural resource and the cost of fish soft roe is relatively cheap. Nucleoprotein supplement prevented the progression of arthritis in of HTLV-1 Tg mice in a dose-dependent fashion. We suggest the radical scavenging effect of nucleoprotein as a putative mode of action. Our results also suggest that nucleoprotein supplement might prove

efficacious in the treatment of RA, delaying the progression of the disease and deferring the need for more traditional clinical interventions. Nucleoprotein therefore holds considerable promise as an alternative medication for RA sufferers.

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