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Beneficial effects of natural eggshell membrane (NEM) on multiple indices of arthritis in collagen-induced arthritic rats

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ABSTRACT

Objectives: This study was performed to evaluate the potential efficacy of natural eggshell membrane (NEM) in collagen-induced arthritic rats, a well-established rodent model of inflammation and rheumatoid arthritis.

Methods: Rats with developing type II collagen-induced arthritis (CIA) were treated once daily by oral gavage on study days –14 to 17 with vehicle or NEM (52 mg/kg body weight). Rats were euthanized on study day 17. Efficacy was assessed by daily ankle caliper measurements, ankle diameter expressed as area under the curve (AUC_{0–17}), and histopathologic evaluation of ankles and knees. Serum biomarkers of cartilage function and inflammation [collagen type II C-telopeptide (CTXII), cartilage oligomeric matrix protein (COMP), and alpha-2-macroglobulin (A2M)] were measured by ELISA.

Results: Treatment with NEM resulted in significant beneficial effects on the daily ankle diameter measurements and ankle diameter AUC. Ankle and knee histopathology scores were significantly reduced (36% and 43% reduction of summed individual histopathology scores for ankle and knee, respectively; $p < 0.05$) toward normal for rats given NEM compared to vehicle controls. The percent reduction of serum CTXII, COMP, and A2M in NEM-treated rats ranged from 30% to 72% ($p < 0.05$).

Conclusions: NEM significantly improved multiple aspects of inflammatory arthritis including inflammation, pannus, cartilage damage, bone resorption, and periosteal bone formation. This study provides further support for the use of CTXII, COMP, and A2M as relevant biomarkers that were responsive to NEM.

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KEYWORDS

A2M; COMP; CTXII;
Methotrexate; Rheumatoid

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory condition, the primary expression of which occurs in the synovial tissues [1]. RA is characterized by polyarticular inflammation which can lead to progressive joint damage [1]. As a result, RA is associated with substantial functional disability, morbidity, and accelerated mortality, all of which pose an enormous and growing societal burden [1]. Although RA is primarily considered a disease of the joints, abnormal systemic immune responses are evident and can cause a variety of extra-articular manifestations such as vasculitis, nodules, and accelerated atherosclerosis [2]. It affects approximately 0.25–1% of the general population worldwide [3,4], although the incidence is higher (5–7%) in Native Americans in the US [2]. RA incidence rates are higher in women than men (2–3-fold), with one study reporting peaks in disease onset at younger ages in women (55–64 years) compared with men (75–84 years) [2,5].

There are a variety of prescription drugs and biologicals approved for use for RA, but these options are often associated with significant side effects and are costly [6–8]. For many disease conditions, natural interventions are preferred by some patients due to their reduced potential for side-

effects and generally lower cost. The most intensively investigated natural products in the context of RA are curcumin, omega-3 fatty acids (i.e. fish oil), gamma linolenic acid, and ginger [9–12]. To efficiently identify and evaluate new candidate interventions for RA, irrespective of whether they are synthetics, biologicals, or natural products, it is imperative to use animal models that reflect some aspects of the clinical pathology and that offer predictive responses.

Collagen-induced arthritis (CIA) in rats is an experimental model of polyarthritis that has been widely used for pre-clinical testing of numerous anti-arthritic agents that are either under preclinical or clinical investigation or are currently used as therapeutics in this disease [13–15]. The hallmarks of this model are reliable onset and progression of robust, easily measurable, polyarticular inflammation, marked cartilage destruction in association with pannus formation, and mild to moderate bone resorption and periosteal bone proliferation. Therapeutic agents that inhibit interleukin-1 (IL-1) production or activity are especially active in this model [16], but other types of anti-inflammatory agents have good to excellent activity [14,15].

Natural eggshell membrane, commercially available as NEM[®], has demonstrated safety and efficacy in multiple

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 Supplemental data for this article can be accessed [here](#).

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clinical trials in relieving joint pain and stiffness in individuals with osteoarthritis [17–20]. NEM has also been investigated for similar benefits in various animal species including a rat model of osteoarthritis [21–25]. Based on the previously reported efficacy of NEM in humans with osteoarthritis and in a rat model of osteoarthritis, the current study was performed to evaluate the potential efficacy of NEM in collagen-induced arthritic rats, a well-established model of RA.

Materials and methods

General

All work was carried out at Bolder BioPATH (Boulder, CO), except for the biomarker assays which were performed at Novus International (Saint Charles, MO). The animal protocol was approved by Bolder BioPATH's Institutional Animal Care and Use Committee (IACUC) for compliance with regulations prior to study initiation (IACUC Protocol #BBP12-002); all procedures complied with all federal (USA) and state (CO and MO) statutes ensuring the humane and ethical treatment of experimental animals.

Materials

Commercially available NEM (lots 8011140, 8011510, 8011910) was provided by ESM Technologies (Carthage, MO). NEM is a natural, food-sourced ingredient obtained from the membrane of chicken eggshells. The NEM matrix contains hyaluronic acid, glycosaminoglycans (GAGS), and collagen (types I, V, and X) along with other proteins (50–70%). Methotrexate and methylcellulose vehicle (Item #M6385) were purchased from Sigma-Aldrich (St. Louis, MO). Porcine type II collagen (Item #20031) was purchased from Chondrex (Redmond, WA). Freund's Incomplete Adjuvant was purchased from Difco (Detroit, MI).

Animals, care, and diet

Female Lewis rats ($n=54$) weighing 115–139 g (mean 127 g) on day –14 were obtained from Charles River Laboratories (Wilmington, MA). Upon arrival, animals were housed 3–4 per cage in shoe-box polycarbonate cages with wire tops, wood chip bedding, and suspended food and water bottles. Animal care including room, cage, and equipment sanitation conformed to the guidelines cited in the Guide for the Care and Use of Laboratory Animals and the applicable standard operating procedures of Bolder BioPATH [26]. Animals were identified by a distinct number at the base of the tail delineating group and animal number. After randomization, all cages were labeled with protocol number, group number, and animal numbers with the appropriate color-coding.

Animals were acclimated for eight days prior to being immunized with type II collagen. An attending veterinarian was on site or on call during the live phase of the study. No concurrent medications were given. During the acclimation and study periods, animals were housed in a laboratory

environment with temperatures ranging 67–76°F and relative humidity of 30–70%. Automatic timers provided 12 h of light and 12 h of dark. Animals were allowed access *ad libitum* to Harlan Teklad Rodent Chow and fresh municipal tap water.

Experimental design

Rats ($n=10$ per group for arthritis induction by collagen injection) were randomized by body weight into treatment groups on study day –14. Once daily oral dosing (gavage) with NEM (52 mg/kg; three different lots evaluated) suspended in 0.5% (w/v) methylcellulose in water or vehicle was initiated, it continued throughout the study. Test article was prepared weekly. This dose of NEM approximates the human equivalent dose (500 mg per day) which has been previously shown to be efficacious in several clinical studies [17,18]. On study day 0, rats (groups 2–6) were anesthetized with isoflurane and injected with 400 μ l of Freund's Incomplete Adjuvant containing 2 mg/ml porcine type II collagen at the base of the tail at two sites (200 μ l per site). On study day 7, rats received an additional injection (i.e. booster) of 100 μ l at one site at the base of the tail. The experimental groups were as follows: Group 1, healthy rats, vehicle control ($n=4$); Group 2, arthritic rats, vehicle control ($n=10$); Group 3, arthritic rats, NEM (lot 8011140) ($n=10$); Group 4, arthritic rats, NEM (lot 8011510) ($n=10$); Group 5, arthritic rats, NEM (lot 8011910) ($n=10$); Group 6, arthritic rats, methotrexate (75 μ g/kg) ($n=10$). Methotrexate served as the reference compound, and was administered by oral gavage from days 0 through 17; the vehicle was administered on days –14 through –1. In the past 25 years, methotrexate has become the most widely used disease-modifying anti-rheumatic drug in humans [27], and is efficacious in animal models of RA including collagen-induced arthritic rodents [14,15]. Methotrexate is most effective and useful as a reference compound when used at a sub-maximal dose in the context of developing RA (i.e. as a prophylactic intervention), where the opportunity exists to administer it for a longer duration without incurring major side effects or toxicity. The ED₅₀ dose range of methotrexate is approximately 60–70 μ g/kg/day in collagen-induced arthritic rats [14,15]. Studies in which rats were dosed with methotrexate for longer periods of time (compared to the duration of exposure here) at a dose of 100 μ g/kg/day have revealed bone marrow hypocellularity, intestinal lesions, and mortality (unpublished data, A. Bendele).

Observations, measurements, and specimens

Rats were weighed on study days –14, –7, 0, 7, and 9–17. Caliper measurements of right and left ankle diameters were taken on study days 9–17. Ankle caliper measurements were made with a Digitrix II micrometer (Fowler & NSK; Newton, MA). Baseline measurements were taken using one ankle with values rounded to one-thousandth of an inch. Measurements were confirmed as clinically normal

(0.260–0.264 in) by comparison with historical values for rats based on a range of body weights. Baseline measurements were then applied to both ankles, and these values remained with the animal so long as the ankle was clinically normal with good definition of all the ankle bones and no evidence of inflammation. On study days –14 and 13, rats were bled by tail vein for serum collection (300 µl). Serum samples were stored frozen at –80°C until shipment on dry ice to Novus International (St. Charles, MO). At necropsy (day 17), rats were anesthetized with Isoflurane and bled by vacutainer through the descending aorta for serum, before being euthanized for tissue collection. Knee lavages were performed on day 17 by injecting 50 µl of saline into each knee followed by repeated flexion and extension. The fluid was removed (pooled from both knees) and centrifuged, and the resulting supernatant was stored frozen at –80°C until shipment on dry ice to Novus (St. Charles, MO). Hind paws were transected at the level of the medial and lateral malleolus, weighed, and placed (with knees) in 10% neutral buffered formalin for microscopy. Livers, spleens, and thymuses were removed, trimmed of extraneous tissue, weighed, and discarded.

Biomarker assays

Samples were processed according to the instructions provided by the manufacturers of the four biomarker kits. ELISA kits were used for the measurement of CTXII (Nordic Bioscience Diagnostics, Herlev, Denmark; serum pre-clinical Cartilaps ELISA, Catalog# 3CAL4000), COMP (MD Biosciences, St. Paul, MN; catalog# A-COMP.96), A2M (Life Diagnostics, West Chester, PA; catalog# 2810-2), and IL-1β [Pierce Biotechnology (a subsidiary of ThermoFisher Scientific), Rockford, IL; catalog# ER2IL1B]. Protein was measured using the Bio-Rad assay kit (Bio-Rad, Hercules, CA; catalog# 5000002).

Morphologic pathology

Histopathology was performed on joints from arthritic rats treated with NEM (52 mg/kg/day; lot 8011510) or vehicle. Historical data obtained by Bolder BioPATH (Boulder, CO) were used for normal controls. Preserved and decalcified (5% formic acid) ankle and knee joints were cut in half longitudinally (ankles) or in the frontal plane (knees), processed through graded alcohols and a clearing agent, infiltrated and embedded in paraffin, sectioned, and stained with toluidine blue by Bolder BioPATH-associated personnel (HistoTox Labs, Boulder, CO). Tissues from all animals were examined microscopically by a board certified veterinary pathologist (Dr. Alison Bendele), and observations were entered into a computer-assisted data retrieval system. Details of the graded scoring system are provided in the Supplementary File 1.

Statistical analyses

Clinical data for ankle joint diameter and ankle score were analyzed by determining the area under the dosing curve

(AUC). For calculation of AUC, the daily measurement of ankle joints (using a caliper) for each rat was entered into Microsoft Excel and the area between the treatment days after the onset of disease to the termination day was computed. Means for each group were determined and % inhibition from arthritis controls was calculated by comparing values for treated and normal animals. Data were analyzed using a one-way analysis of variance (ANOVA) for measured parameters, or a Kruskal–Wallis non-parametric test for scored parameters, along with the appropriate multiple comparison post-tests. Where appropriate, select histopathology parameters were also compared to vehicle controls using a Mann–Whitney *U* test. Statistical analysis and figure preparation were performed on untransformed data using GraphPad Prism (version 5; San Diego, CA). Significance for all tests was set at $p < 0.05$. Percent inhibition of AUC and other parameters where indicated was calculated using the following formula: % Inhibition = $B/A \times 100$, where A = mean healthy control – mean disease control and B = mean treated – mean disease control. This method takes into account non-zero values for the healthy control group.

Results

Effects of NEM on ankle swelling

Previous studies in adult patients with osteoarthritis with NEM have established that the efficacious daily dose is 500 mg [18,19]. To identify the corresponding efficacious dose of NEM in the CIA-induced rat model, we used two independent approaches. First, we calculated the predicted efficacious dose in rats based on the body surface area method [28]. This method is more accurate compared to the simple, straight conversion based on body weight. The equation for dose conversion between species is given as:

$$\begin{aligned} \text{Human Equivalent Dose (mg/kg)} \\ &= \text{Animal Dose (mg/kg)} \\ &\quad \times \text{Animal Km factor/Human Km factor} \end{aligned}$$

where Km is a correction factor reflecting the relationship between body weight and body surface area [28]. Assuming a 60 kg adult and Km values of 6 and 37 for rat and human [28], respectively, we determined that the rat-equivalent efficacious daily dose of NEM was 51.4 mg/kg. To confirm this calculated dose was efficacious, we conducted two preliminary dose–response experiments evaluating the effect of three different doses of NEM on ankle swelling. Our dose–response-data confirmed that a daily dose of (a) 26 mg/kg was not efficacious, (b) 52 mg/kg was efficacious, and that (c) 103 mg/kg provided no superior efficacy beyond that observed for 52 mg/kg (Supplementary File 2). For subsequent experiments, we chose to use a daily oral dose of 52 mg/kg.

Collagen-induced arthritis produced a marked increase in ankle diameter compared to healthy controls, an effect that was evident by day 10 (Figure 1A). Oral administration of each lot of NEM attenuated this increase, beginning on day

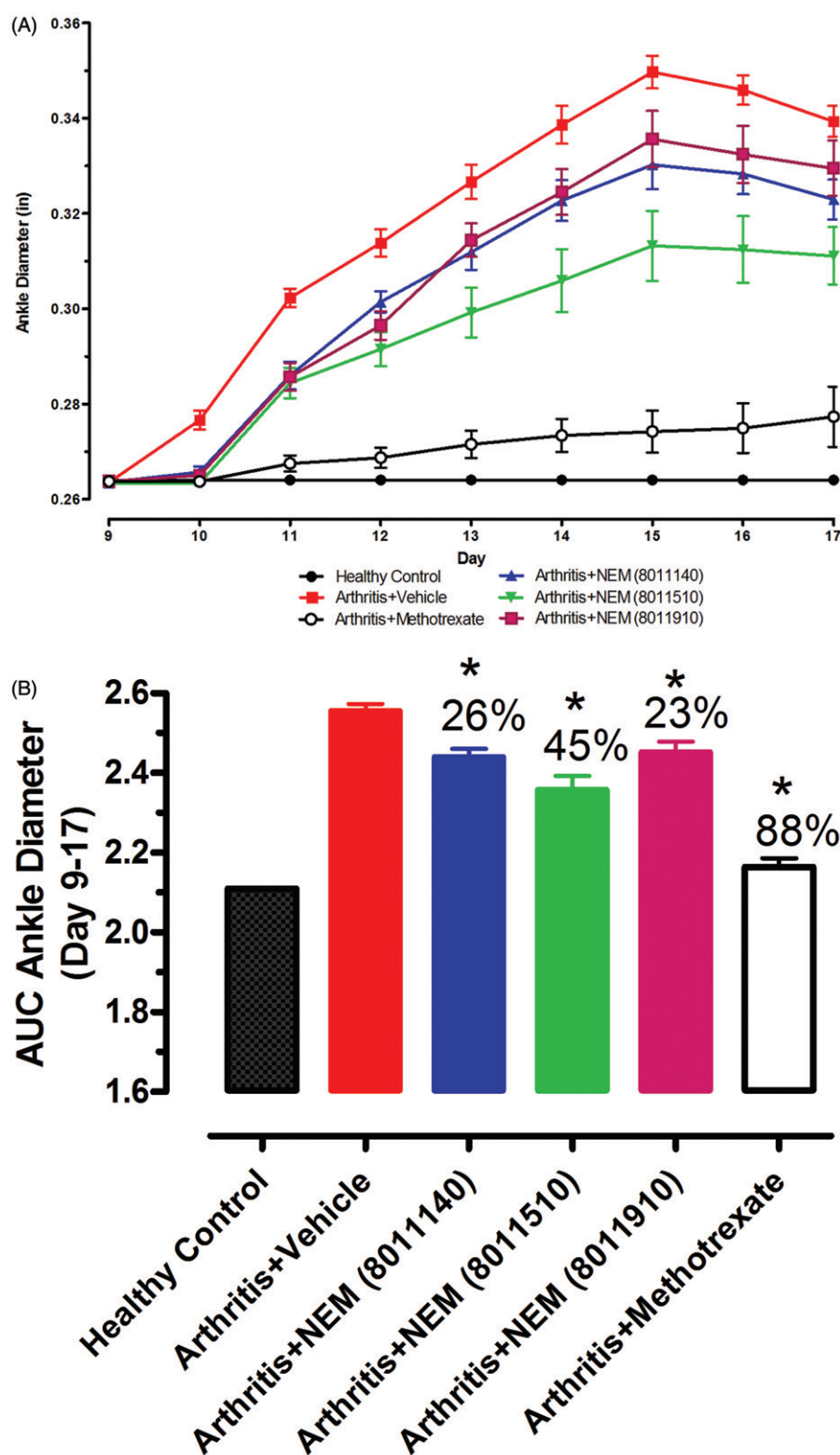


Figure 1. Effect of NEM on ankle diameter. Ankle measurements (Panel a) and AUC (Panel b) calculations were performed as described in Materials and Methods. Numbers above bars in Panel b indicate % inhibition calculated as described in Materials and Methods. Asterisks indicate statistical significance, defined as $p < 0.05$ vs arthritis vehicle.

10 and persisting through the remainder of the study. Daily ankle diameter measurements were significantly reduced toward normal for rats treated with NEM-8011140 ($p < 0.05$ on days 10–13 and 15), NEM-8011510 ($p < 0.05$ on days 10–17), and NEM-8011910 ($p < 0.05$ on days 10–12). As expected, methotrexate was effective at reducing ankle

swelling throughout the study ($p < 0.05$ on days 10–17). Ankle diameter expressed as AUC was significantly reduced ($p < 0.05$) toward normal for rats administered NEM-8011140 (26% reduction), NEM-8011510 (45%), NEM-8011910 (23%), and methotrexate (88%), as compared to the arthritis vehicle control (Figure 1B).

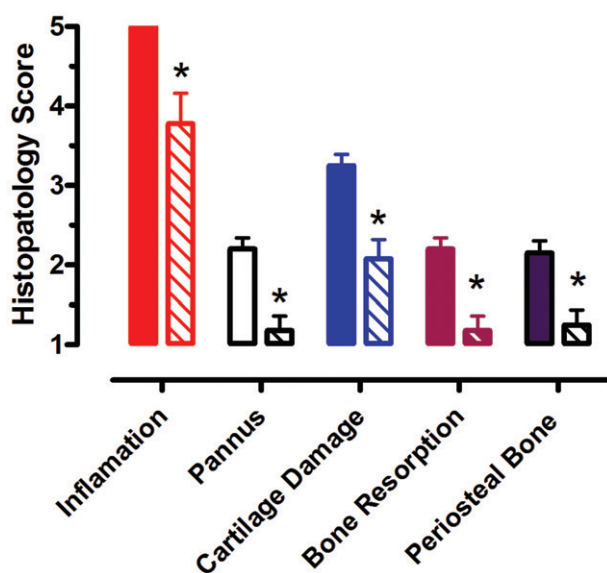


Figure 2. Effects of NEM on individual ankle histopathology scores. Collagen arthritic ankles were given scores of 0–5 for inflammation, pannus formation, cartilage damage, bone resorption, and periosteal new bone formation according to the criteria indicated in Supplementary Information. Vehicle control, solid bars; NEM-8011510, hatched bars. Asterisks indicate statistical significance, defined as $p < 0.05$ vs arthritis vehicle.

Effects of NEM on morphologic pathology of ankle joints

Histopathology was performed on ankle and knee joints from vehicle control arthritic rats and rats treated with NEM-8011510. Historical data archived at Bolder BioPATH were used for healthy control rats. All vehicle-treated disease control rats had severe synovitis and periarticular inflammation in both ankle joints with minimal to moderate pannus formation, bone resorption, and periosteal bone formation, and mild to marked cartilage damage. Mean periosteal bone width was $409.50 \mu\text{m}$ (data not shown). As shown in Figure 2, all ankle histopathology parameters were significantly ($p < 0.05$) reduced toward normal for arthritic rats treated with NEM-8011510. Rats treated with NEM-8011510 had significantly reduced inflammation (25%), pannus formation (47% reduction), cartilage damage (36%), bone resorption (47%), and periosteal bone formation (42%), as compared to the arthritis vehicle control. Overall, NEM produced a 36% reduction ($p < 0.05$) in the summed histopathological score, along with an approximate 40% reduction ($p < 0.05$) in periosteal bone width. Representative photomicrographs of ankles with the approximate mean summed histopathological scores for each group are shown in Figure 3; NEM-8011510 produced marked improvements in each of the arthritis-induced responses (see figure legend for detailed description).

Effects of NEM on morphologic pathology of knee joints

All vehicle control animals had marked to severe synovitis and periarticular inflammation in at least one knee joint with minimal to moderate pannus formation, none to moderate bone resorption, and minimal to marked cartilage damage. As shown in Figure 4, all knee histopathology

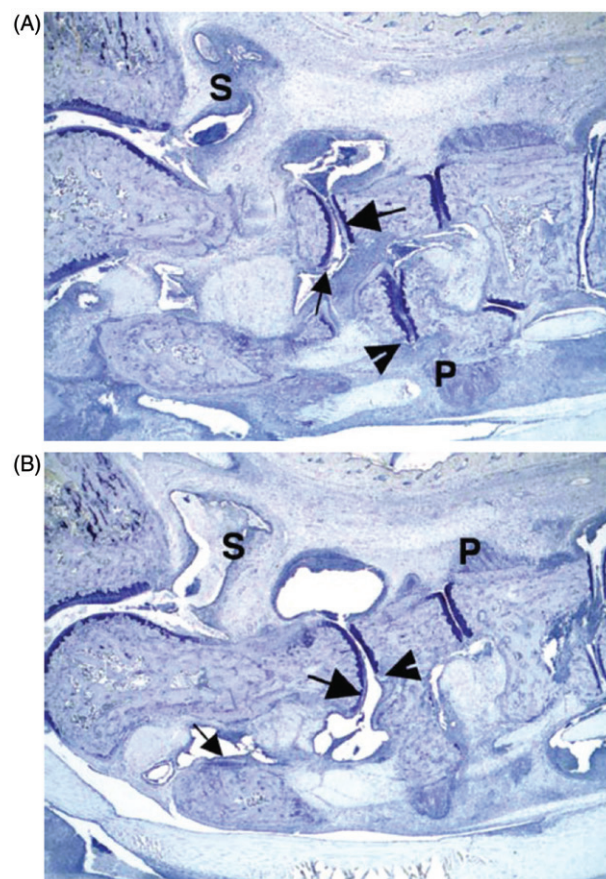


Figure 3. Effects of NEM on morphologic pathology ankle joint. Panel A: Ankle from an arthritis vehicle control animal (with the approximate mean summed score for the group; animal # 7, left ankle) has severe inflammation (S) and moderate cartilage damage (large arrow) with mild pannus (small arrow) and bone resorption (arrowhead), as well as moderate periosteal bone formation (P). Panel B: Ankle from an arthritic animal treated with NEM-8011510 (with the approximate mean summed score for the group; animal #7, left ankle) has marked inflammation (S) and mild cartilage damage (large arrow) with minimal pannus (small arrow) and bone resorption (arrowhead), as well as mild periosteal bone formation (P). Each slide was evaluated at $16\times$ magnification.

parameters were significantly ($p < 0.05$) reduced toward normal for arthritic rats treated with NEM-8011510. Rats treated with NEM-8011510 had significantly reduced inflammation (40%), pannus formation (43% reduction), cartilage damage (43%), and bone resorption (48%) as compared to the arthritis vehicle control. Overall, NEM produced a 43% reduction ($p < 0.01$) in the summed histopathological score. Representative photomicrographs of knees with the approximate mean score for each group are shown in Figure 5; NEM-8011510 produced marked improvements in each of the arthritis-induced responses (see figure legend for detailed description).

Effects of NEM on biomarkers

Collagen type II C-telopeptide (CTXII), cartilage oligomeric matrix protein (COMP), and alpha-2-macroglobulin (A2M) were measured in serum samples from healthy rats, arthritic rats, arthritic rats provided with NEM-8011510, and arthritic rats treated with methotrexate (Figure 6A–C). There were no significant inter-group differences in any of the biomarkers at day –14, prior to collagen injection and NEM

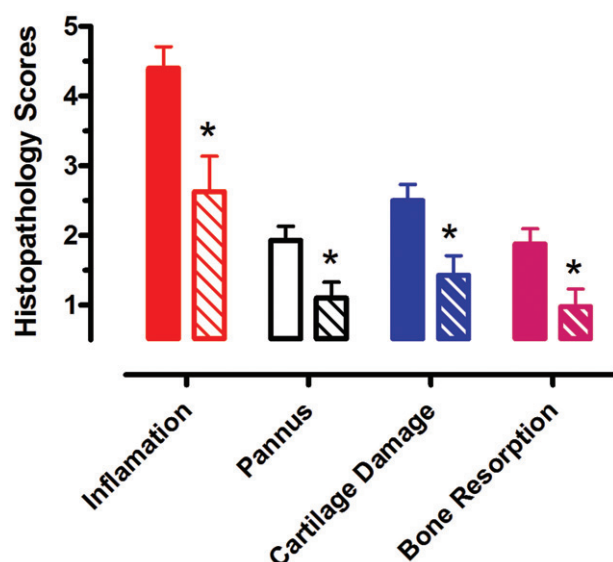


Figure 4. Effects of NEM on individual knee histopathology scores. Collagen arthritic knees were given scores of 0–5 for inflammation, pannus formation, cartilage damage, and bone resorption according to the criteria indicated in Supplementary Information. Vehicle control, solid bars; NEM-8011510, hatched bars. Asterisks indicate statistical significance, defined as $p < 0.05$ vs arthritis vehicle.

administration (data not shown). CTXII was measured at days 13 and 17. In arthritic rats, CTXII was significantly elevated (2.3–3.6-fold, respectively) at both time points compared to healthy controls. The percent inhibition of serum CTXII in NEM-8011510-treated rat was 32% ($p < 0.05$) at day 13, and 30% ($p < 0.05$) at day 17. COMP was measured at days 13 and 17. In arthritic rats, COMP was significantly elevated (approximately 1.9-fold) at both time points compared to healthy controls. The percent inhibition of serum COMP in NEM-8011510-treated rat was 40% ($p < 0.05$) at day 13, and 27% (not significant) at day 17. A2M was measured at days 13 and 17. In arthritic rats, A2M was significantly elevated (4.7–6-fold) at both time points compared to healthy controls. The percent inhibition of serum A2M in NEM-8011510-treated rat was 72% ($p < 0.05$) at day 13, and 64% ($p < 0.05$) at day 17. IL-1 β was measured in fluid obtained following knee lavage on day 17 as described in Materials and Methods (Figure 6d). In arthritic rats, IL-1 β was significantly elevated (approximately 19-fold) compared to healthy controls. The percent inhibition of IL-1 β in NEM-8011510-treated rat was 49% ($p < 0.05$) at day 17.

Effects of NEM on body and organ weights

From study days –14 through 17, healthy rats had mean body weight gain of 69.8 g, and vehicle control arthritic rats had mean body weight gain of 34.8 g. Body weight gain was significantly increased toward normal for rats given NEM (lot 8011510; 34% increase; $p < 0.05$) and methotrexate (77%; $p < 0.05$) compared to vehicle controls (Supplementary File 3). Final paw weights were significantly reduced toward normal for rats given 8011510 (33% reduction) and methotrexate (79%) as compared to vehicle controls (Supplementary File 4). Liver weights relative to body weight were not significantly affected for rats in any

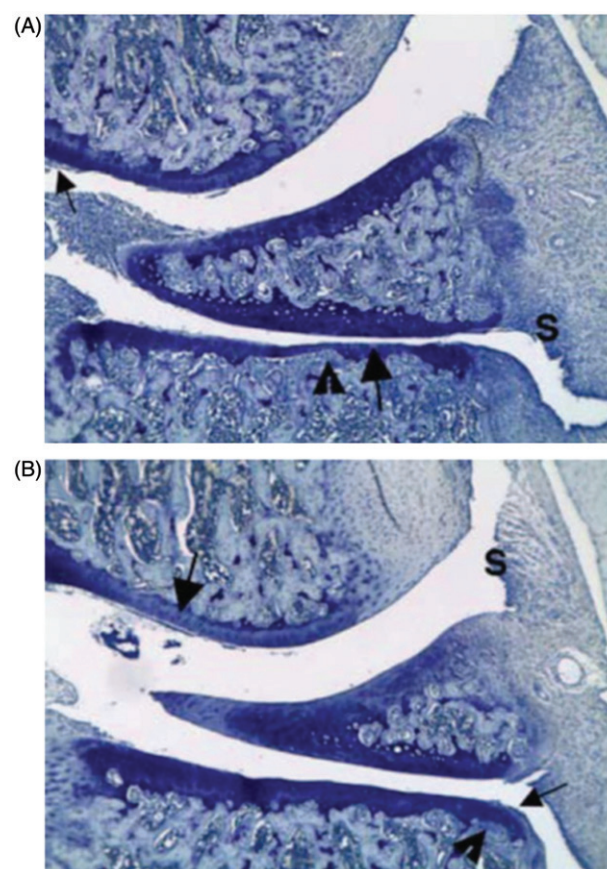


Figure 5. Effects of NEM on morphologic pathology knee joint. Panel A: Knee from a arthritis vehicle control animal (with the approximate mean summed score for the group; animal 1, right knee) has severe inflammation (S) with mild pannus (small arrow), cartilage damage (large arrow), and bone resorption (arrowhead). Panel B: Knee from an arthritic animal treated with NEM-8011510 (with the approximate mean summed score for the group; animal #9, right knee) has moderate inflammation (S) and mild cartilage damage (large arrow) with very minimal pannus (small arrow) and bone resorption (arrowhead). Each slide was evaluated at 50 $\times$ magnification.

treatment group as compared to vehicle controls (Supplementary File 3). Spleen weights relative to body weight were significantly reduced for rats treated with methotrexate as compared to arthritis vehicle control (Supplementary File 4). Thymus weights relative to body weight were not significantly affected for rats in any treatment group as compared to vehicle control (Supplementary File 4).

Discussion

The results presented here indicate that NEM exerted significant beneficial effects on inflammation (judged by degree of ankle swelling and histopathology score) and joint pathology (judged by histopathology of the ankle and knee) in collagen-induced arthritic rats. The NEM-mediated reduction in ankle swelling measurement was observed as early as day 10 post-collagen injection and persisted through the end of the study. Overall, NEM produced an approximate 25–45% reduction in ankle diameter AUC (days 10–17) compared to the vehicle-treated rats. Histopathology evaluation of the ankle at study termination indicated that NEM produced significant reductions compared to vehicle-treated

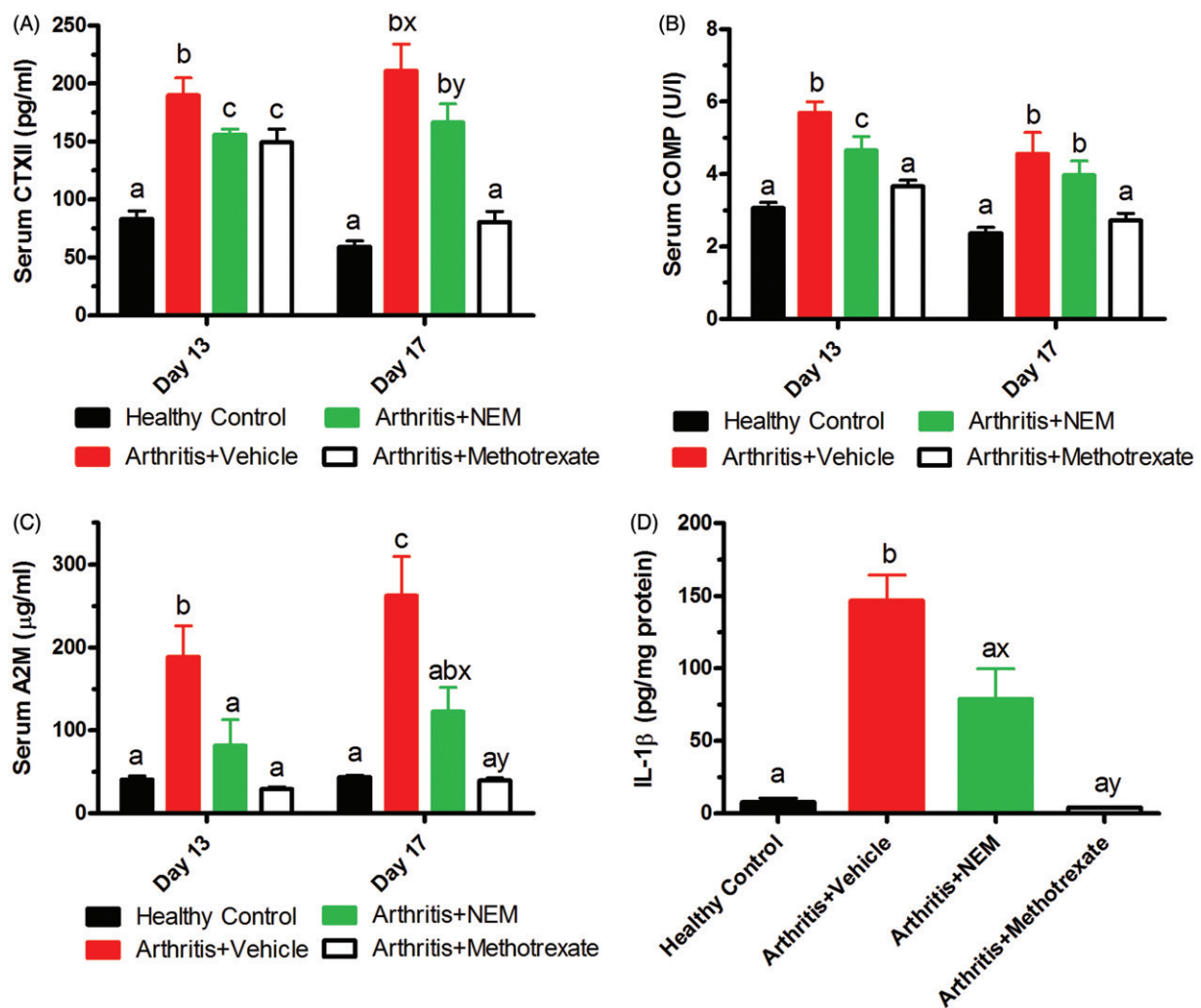


Figure 6. Effects of NEM on biomarkers CTXII, COMP, A2M, and IL-1 β . Biomarkers were measured as described in Materials and Methods. CTX II, COMP, and A2M were measured in serum samples, and IL-1 β was measured in knee lavage fluid. Bars with different letters indicate that they were statistically different at $p < 0.05$.

control rats in the individual scores, including those for inflammation (−25%), pannus (−47%), cartilage damage (−36%), bone resorption (−47%), and periosteal bone formation (−42%). Impressively, NEM produced an approximate 36% reduction in the overall histopathology score for the ankle compared to the vehicle control. In addition, direct microscopic measurement of periosteal bone formation indicated that NEM reduced this compensatory response by approximately 43%.

The beneficial effects of NEM were not limited to the ankle, as analogous effects were observed in the knee. Histopathology evaluation of the knee at study termination indicated that NEM produced significant reductions compared to vehicle-treated control rats in the individual scores, including those for inflammation (−40%), pannus (−43%), cartilage damage (−43%), and bone resorption (−48%). NEM produced an approximate 43% reduction in the overall histopathology score for the ankle compared to the vehicle control. Under the conditions of this study design, NEM was well tolerated with no obvious intervention-related adverse effects. These results obtained in a rat model of RA add to the growing body of evidence supporting the efficacy

of NEM in arthritic rats and in humans with osteoarthritis [18,19,25,29].

As cartilage degrades, fragments of CTX-II are released into circulation and subsequently secreted into urine. CTX-II is an intensely studied biomarker for cartilage degradation and disease progression, including RA, osteoarthritis, and other inflammatory diseases of the joints. In patients with RA, an increase in CTXII has been reported to be temporally linked to the progression of arthritis severity [30]. In patients with early RA (through two years), multivariate analyses indicated that a model including CTXII (along with matrix metalloproteinase-3; MMP-3) provided the best prediction of radiographic progression at entry [31]. In patients with RA treated aggressively with combination-therapy [COBRA regimen, including temporary high-dose prednisolone, temporary low-dose methotrexate, and sulfasalazine] or mild-monotherapy (sulfasalazine), the individual CTXII response measured after three months of therapy in patients who had increased CTXII levels at baseline independently predicts five-year radiographic progression [32].

COMP is another well-studied biomarker of cartilage breakdown and turn-over [33,34], and has been proposed as

a useful biomarker in arthritis [35–37]. There are numerous reports of COMP being elevated in patients with RA in serum and synovial fluid, and it has been suggested to be a marker of joint damage progression with prognostic value [38,39]. Furthermore, in patients with RA, COMP is responsive (i.e. decreases) to treatment with numerous biological and pharmaceutical interventions [40–43].

NEM has been shown to significantly reduce the levels of both CTXII and COMP in monoiodoacetate (MIA)-induced arthritic rats, in combination with organic trace minerals (Zn, Cu, and Mn) chelated to 2-hydroxy-4-(methylthio)butanoate (Mintrex®) and when provided as a single intervention [21,25]. In agreement with previous studies, NEM reduced the level of serum CTXII by day 13 and this effect persisted through the end of the study. NEM also reduced the level of COMP, but the effect was statistically significant at day 13 only. As reported by Sim et al., a higher dose of NEM was required (400 mg/kg/day) to reduce COMP in MIA arthritic rats than the dose administered in this study (52 mg/kg/day, representing the human equivalent dose of 500 mg per day). These findings suggest that either the MIA arthritic rat is a more severe model of arthritis, or that COMP is less sensitive to NEM, thereby exhibiting a dose response curve that is right-shifted [25].

A2M is a member of the alpha macroglobulin family, and functions as a broad-spectrum proteinase inhibitor [44]. It is synthesized and secreted by the liver as a compensatory response to chronic inflammation. Interestingly, A2M forms complexes and is able to associate with the matrix metalloproteinases (MMPs), which are elevated in many chronic inflammatory diseases such as RA, and is considered as a potential biomarker of MMP activity [45]. A2M is a sensitive inflammatory biomarker for RA [45–47]. Compared to CTXII and COMP, A2M was the most sensitive to the inhibitory effects of NEM and methotrexate. NEM reduced A2M by 72% at day 13 and 64% at day 17, while methotrexate essentially reduced A2M to the level of the healthy controls.

Both IL- α and IL-1 β are validated molecular targets for several rheumatological diseases [16,48]. Anakinra (Kineret®) is the recombinant human form of the naturally occurring IL-1 receptor antagonist (IL-1Ra) and is approved for use for RA in the US and some other world areas [49]. In the present study, we found that IL-1 β was elevated by about 19-fold in synovial fluid obtained from the knee joints of arthritic rats, and reduced by approximately 50% in NEM-treated rats.

Animal models of arthritis are used to study pathogenesis of disease and to evaluate potential anti-arthritic drugs for clinical use [15,50]. Therefore, morphological similarities to human disease and capacity of the model to predict efficacy in humans are important criteria in model selection. Animal models of RA with a proven track record of predictability for efficacy in humans include: rat type II collagen arthritis, mouse type II collagen arthritis, rat adjuvant arthritis, and antigen-induced arthritis in several species [14–16]. Agents currently in clinical use (or trials) that are active in these models include: corticosteroids, methotrexate, non-steroidal

anti-inflammatory drugs, cyclosporin A, leflunomide, IL-1Ra, and soluble TNF receptors.

In addition to prescription pharmaceutical and biological interventions, several natural products have been evaluated in collagen-induced arthritic rats. Most recently, the beneficial effects of δ -tocotrienol (10 mg/kg) on reducing paw edema and improving histopathological features when administered on days 25 through 50 post-collagen injection were reported [51]. Similarly, oral administration of the probiotic *Lactobacillus casei* improved the individual histopathology scores and overall arthritis score, along with reducing the levels of several inflammatory cytokines [52]. The authors suggested that the efficacy of this probiotic was related to inhibition of cyclooxygenase-2. A combination of glucosamine HCl/chondroitin sulfate/manganese ascorbate improved the histopathological scores, but failed to alter T-cell proliferation and antibody production to bovine type-II collagen, indicating that its effects were not due to alteration of the antigen-specific immune response [53]. Finally, administration by gavage of the total alkaloid fraction of *Tripterygium wilfordii* Hook F, a Traditional Chinese Medicine, for one month significantly reduced paw swelling, suppressed articular cartilage degeneration, and reduced the level and expression of several inflammatory cytokines [54]. *Tripterygium wilfordii* Hook F also holds promise as a medical botanical based on a recent report evaluating its safety and efficacy, alone and in combination with methotrexate, in a double-blind placebo-controlled study in subjects with RA [55].

The manner in which NEM exerts beneficial activity *in vivo* has been the subject of a number of prior studies. NEM has been shown to have direct immunomodulatory effects wherein an NEM extract reduced various pro-inflammatory cytokines (e.g. TNF- α , IFN- γ) in mitogen-activated human immune cells *in vitro* [56]. It was recently proposed that NEM may also have indirect immunomodulatory effects via NEM-mediated activation of NF- κ B, a pro-inflammatory transcription factor, through an oral tolerance mechanism initiated in the gut-associated lymphoid tissue (GALT) [57]. Oral tolerance has been thoroughly investigated for nearly 50 years and numerous studies have demonstrated that oral suppression of the CIA rat model occurs via oral tolerance [58–60]. Therefore, this study provides additional evidence in support of indirect immune modulation by NEM. Lastly, it has also been demonstrated via radiolabeling that approximately 40% of eggshell membrane gets digested and absorbed while the remaining ~60% is excreted in the feces [61]. Taken together, these studies provide reasonable evidence that NEM likely works via a bimodal mechanism of action. That is, direct immune modulation from absorbed, bioavailable peptides, and/or glycopeptides and indirect immune modulation from unabsorbed protein and/or glycoprotein fragments.

It is also important to note that the present study is the first evidence, to our knowledge, that a Type I collagen-containing composition such as NEM can ameliorate Type II CIA. This indicates that there is sufficient homology in the tertiary protein structure of the two types of collagen to nevertheless illicit immune suppression. By inference, it

would be expected that NEM and other Type I collagen compositions would similarly suppress the detrimental immune response to Type II collagen-containing cartilage fragments that ensues in naturally occurring forms of arthritis, particularly RA and OA. This undoubtedly speaks to the mechanism by which NEM affects the clinical efficacy found to date [17–19].

This study had a number of strengths and limitations. The primary strengths of the study included the use of the CIA-induced arthritic rat, a well validated rat model of inflammatory arthritis with a proven track record of predictability for efficacy [15,62]. A wide variety of approved prescription medications and biological therapies exhibit efficacy in this model. The nutritional intervention NEM can now be added to this list. Another strength of the study was the use of the human equivalent dose of NEM. Too often data from animal studies are obtained using supra-pharmacological dosing regimens bearing little, if any, relevance or relationship to the dose required for clinical efficacy. This is especially true for studies evaluating nutritional interventions. The rationale for the daily dose selected in the present study was based on two key factors: (1) the calculation of the animal dose based on the efficacious human equivalent dose (500 mg efficacious in humans, corresponding to 8.33 mg/kg for a 60 kg adult) using the body surface area method as described by Reagan-Shaw et al. [28] and (2) two dose/response studies. In each case, we determined that the appropriate daily dose for rats to be 52 mg/kg.

An additional strength of the study was the use of validated, complementary measurements including physical, histological, and biochemical assessments. Each of the study endpoints has been employed successfully in many previous non-clinical assessments of both pharmacological and nutritional interventions in various rat models. In particular, the use in human studies of biomarkers for both safety and efficacy is growing at a rapid pace, and the use of CTXII, COMP, and A2M extends their applicability to the collagen-induced arthritic rat model. Finally, the use of a positive reference standard, methotrexate ensured that during the course of all experiments, our animals were sensitive and responsive. In this regard, NEM was approximately 50% as efficacious as methotrexate at reducing the severe inflammation in this model of RA. This suggests that NEM could offer advantages over methotrexate in a clinical context, due to its documented efficacy and superior side effect profile [17–19]. Low-dose methotrexate is associated with severe adverse side effects, including hepatotoxicity, pulmonary tissue damage, myelosuppression, and impaired renal function which often cause patients to discontinue its use [63–67].

The primary limitation of this study was the limited selection of biomarkers. Each of the biomarkers that we selected for this study (CTXII, COMP, A2MG, and IL-1 β) has been reported to be associated with RA in pre-clinical and clinical studies (see above). IL-1 β is validated molecular target for the treatment of RA in humans [48]. Two major biomarkers that were not included in this study were TNF- α and MMP-3. TNF- α has long been a validated molecular target for the treatment for RA, and there are numerous anti-TNF- α agents currently approved for clinical use

throughout the world [68,69]. In addition, MMP-3 is a highly relevant biomarker in the context of RA, although there are no currently available agents that specifically target MMP-3 activity. Several recent studies in patients with RA have reported that baseline elevated MMP-3 is predictive of radiographic progression [31], and that treatment of patients with RA with either methotrexate or iguratimod lowers MMP-3 [31,70–75].

Conclusions

In conclusion, these results demonstrate the efficacy of NEM in a well validated rat model of inflammatory arthritis. NEM exhibited beneficial effects on multiple aspects of the disease including inflammation, pannus, cartilage damage, bone resorption, and periosteal bone formation. In addition, this study provides further support for the use of CTXII, COMP, A2M, and IL-1 β as relevant biomarkers that were responsive to NEM (and methotrexate). Taken together, these results support the beneficial effects of NEM on key pathologies of arthritis including inflammation and cartilage degradation.

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Conflict of interest

KJW, CAA, and JLE are employees of Novus International (St. Charles, MO) which co-markets NEM® (with ESM Technologies, Carthage, MO) through its Stratum Nutrition business unit. KJR is an employee of ESM Technologies which manufactures and co-markets NEM with Stratum Nutrition. AB is an employee of Bolder BioPATH (Boulder, CO), an independent contract research organization that was contracted by Novus International to perform this study. Funding for this study was provided by Novus International (St. Charles, MO) and ESM Technologies (Carthage, MO). These relationships are provided in the spirit of transparency; none of the authors benefit financially (e.g. royalties, commissions, bonuses, etc.) from the sale of any intervention used in this study.

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