

Research Summary

Research & Patent Portfolio on NEM® brand eggshell membrane



3X JOINT DEFENSE

PAIN REDUCTION*

STIFFNESS REDUCTION*

CARTILAGE PROTECTION*

*following exercise



Research & Patent Portfolio on NEM® brand eggshell membrane

NEM® is the original, #1 branded eggshell membrane ingredient with multiple published safety and efficacy studies – the most researched eggshell membrane ingredient for joint health. *In vitro*, *in vivo*, pilot and RCT studies accompany a comprehensive safety profile with patents for manufacturing and use. Recent trials conducted in exercising, healthy individuals support NEM's ability to help reduce exercise-induced pain and stiffness, as well as support cartilage protection. NEM contains naturally occurring nutrients essential for healthy joint cartilage and tissue including glucosamine, chondroitin and hyaluronic acid, as well as, collagen, peptides and other beneficial proteins – all working together for one complete joint health solution.*

Published Research in Peer-reviewed Journals:

1) Damjanov *et al.* (2019) NEM® Brand Eggshell Membrane in the Treatment of Pain and Stiffness Associated with Knee Osteoarthritis: An Open Label Clinical Study. *Journal of Arthritis*, 8(5):1000287.

2) Eskiurt *et al.* (2019) Efficacy and Safety of Natural Eggshell Membrane (NEM®) in Patients with Grade 2/3 Knee Osteoarthritis: A Multi-Center, Randomized, Double-blind, Placebo-Controlled, Single-crossover Clinical Study. *Journal of Arthritis*, 8(4):1000285.

3) Ruff KJ, Back M, Morrison D, Duncan SA. (2019) Development of a Novel Clinical Trial Design to Evaluate the Effects of Joint Therapeutics on Cartilage Turnover in Healthy Subjects. *Journal of Novel Physiotherapies*, 9(3):1000415.

4) Ruff KJ, Theodosakis J, Morrison D, Duncan SA, Back M, and Aydogan C. (2018) Eggshell Membrane: Beneficial effects of natural eggshell membrane versus placebo in exercise-induced joint pain, stiffness, and cartilage turnover in healthy, postmenopausal women. *Clinical Interventions in Aging*, 13:285-295.

5) Wedekind KJ, Ruff KJ, Atwell CA, Evans JL and Bendele AM. (2016) Beneficial Effects of Natural Eggshell Membrane (NEM) on Multiple Indices of Arthritis in Collagen-Induced Arthritic Rats. *Modern Rheumatology*, 27(5):838-848.

6) Ruff KJ, Kopp KJ, Von Behrens P, Lux M, Mahn M and Back M. (2016) Effectiveness of NEM® brand eggshell membrane in the treatment of suboptimal joint function in dogs: a multicenter, randomized, double-blind, placebo-controlled study. *Veterinary Medicine: Research & Reports*, 7:113-121.

7) Brunello E and Masini A. (2016) NEM® Brand Eggshell Membrane Effective in the Treatment of Pain and Stiffness Associated with Osteoarthritis of the Knee in an Italian Study Population. *International Journal of Clinical Medicine*, 7:169-175.

- 8)** Sim BY, Bak JW, Lee HJ, Jun JA, Choi HJ, Kwon CJ, Kim HY, Ruff KJ, Brandt K and Kim DH. (2015) Effects of natural eggshell membrane (NEM) on monosodium iodoacetate-induced arthritis in rats. *Journal of Nutrition and Health*, 48(4):310-318.
- 9)** Ruff KJ, Durham PL, O'Reilly A and Long FD. (2015) Eggshell membrane hydrolyzates activate NF- κ B *in vitro*: possible implications for in vivo efficacy. *Journal of Inflammation Research*, 8:49-57.
- 10)** Danesch U, Seybold M, Rittinghausen R, Treibel W and Bitterlich N. (2014) NEM Brand Eggshell Membrane Effective in the Treatment of Pain Associated with Knee and Hip Osteoarthritis: Results from a Six Center, Open Label German Clinical Study. *Journal of Arthritis*, 3(3):136.
- 11)** Ruff KJ and DeVore DP. (2014) Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Modern Research in Inflammation* 3(1):19-25.
- 12)** Benson KF, Ruff KJ and Jensen GS. (2012) Effects of Natural Eggshell Membrane (NEM) on Cytokine Production in Cultures of Peripheral Blood Mononuclear Cells: Increased Suppression of Tumor Necrosis Factor- α Levels After *In Vitro* Digestion. *Journal of Medicinal Food*, 15(4):360-368.
- 13)** Ruff KJ, Endres JR, Clewell AE, Szabo JR and Schauss AG. (2012) Safety evaluation of a natural eggshell membrane-derived product. *Food and Chemical Toxicology*, 50:604-611.
- 14)** Ruff KJ, Winkler A, Jackson RW, DeVore DP and Ritz BW. (2009) Eggshell Membrane in the Treatment of Pain and Stiffness from Osteoarthritis of the Knee: A Randomized, Multicenter, Double Blind, Placebo Controlled Clinical Study. *Clinical Rheumatology*, 28:907-914.
- 15)** Ruff KJ, DeVore DP, Leu MD and Robinson MA. (2009) Eggshell Membrane: A Possible New Natural Therapeutic For Joint & Connective Tissue Disorders. Results From Two Open-label Human Clinical Studies. *Clinical Interventions in Aging*, 4:235-240.

Patent Portfolio:

US 10,328,104 "Methods for Treating NF- κ B Dysregulation in a Host in Need Thereof Using Eggshell Membrane Compositions" (2019). This patent covers the use of eggshell membrane to treat conditions of the gastrointestinal, cardiovascular, nervous, and pulmonary systems that involve immune dysregulation (via NF- κ B).

US 9,983,214 "Method For Evaluating Articular Joint Therapeutics" (2018). This patent covers the use of the cartilage degradation biomarker CTX-II to evaluate the chondroprotective effect of joint therapeutics through an exercise-induced clinical model in either healthy or diseased individuals.

US 8,580,315 "Anti-inflammatory Activity of Eggshell Membrane and Processed Eggshell Membrane Preparations" (2013). This patent covers the reduction of serum pro-inflammatory cytokines by systemically administering eggshell membrane, processed membrane preparations, membrane isolates and combinations thereof.

US 7,017,277 "Vacuum Treatment of an Input Stream Without Ruining Delicate Output Fractions" (2006). This patent covers the separation of shells and membrane.

US 6,946,551 "Preparation of Hyaluronic Acid from Eggshell Membrane" (2005). This patent covers the preparation of hyaluronic acid containing eggshell membrane products.

NEM® Brand Eggshell Membrane in the Treatment of Pain and Stiffness Associated with Knee Osteoarthritis: An Open Label Clinical Study

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Abstract

Objective: NEM® Brand Eggshell Membrane contains collagen and glycosaminoglycans that have beneficial effects in the treatment of Osteoarthritis (OA). A single-center, open-label clinical study was conducted to evaluate the efficacy and safety of NEM® in management of pain and stiffness associated with knee OA.

Methods: Seventy subjects with knee OA received oral NEM® 500 mg once daily for 60 days. The primary outcome measure was to evaluate the effectiveness of NEM® in reducing pain and stiffness associated with knee OA. The primary endpoints were the change in Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), Visual Analog Scale for Pain (VAS pain) and Lequesne Algofunctional Index measured after 10, 30 and 60 days of NEM® supplementation.

Results: NEM® treatment resulted in reduction of WOMAC overall scores at 10 days (16.6%, $p=0.012$), at 30 days (31.8%, $p<0.0001$) and at 60 days (46.7%, $p<0.0001$) post-treatment compared to baseline values. VAS pain was reduced at 10 days (19.6%, $p<0.0001$), at 30 days (31.8%, $p<0.0001$) and at 60 days (49.0%, $p<0.0001$). Overall Lequesne scores were reduced at 10 days (11.2%, $p=0.0002$), at 30 days (24.0%, $p<0.0001$) and at 60 days (36.8%, $p<0.0001$). In a Global Assessment, 68.6% of patients and 78.6% physicians rated the efficacy of NEM® as excellent or good. Three mild and transient, and no serious adverse events were reported.

Conclusions: NEM® supplementation resulted in rapid and significant reduction of joint pain and stiffness (at 10 days) which were further improved at 60 days. NEM® treatment was safe and well tolerated.

Keywords: Osteoarthritis; Pain; Stiffness; Natural eggshell membrane (NEM); Knee; Dietary supplement; Glycosaminoglycans

Introduction

Osteoarthritis (OA) is a degenerative disease characterized by joint pain and stiffness that can cause physical dysfunction and decreased quality of life. OA is a common disease that occurs most often in people over 50 years of age, but also in younger population. The cartilage of articular joints is primarily affected in OA and the knee is one of the most commonly affected joints. The structural changes of articular cartilage, synovial membrane and subchondral bone are due to a combination of risk factors, including aging, obesity, being female, genetics and joint injury. In knee OA, the cartilage of knee joint gradually roughens, becomes thin or wears away causing bone rubbing on bone and pain. OA develops slowly and the joint pain and stiffness usually worsen as the disease progresses [1]. Inflammation is involved in the pathogenesis of OA. Synovitis is common in early and advanced OA and has been associated with knee pain and swelling and progression of cartilage degeneration. Synovium in OA becomes infiltrated by inflammatory cells and increased local levels of pro-inflammatory cytokines such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and IL-6 produced by these cells can enhance cartilage degradation or induce bone resorption [2-4].

Medication-based therapies in OA comprise different drugs, including analgesics (e.g. paracetamol, hydrocodone) or non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ibuprofen, celecoxib, etc.), alone or in combination. These therapies have shown limited effectiveness in clinical studies or may have significant and serious side effects [5-8].

NEM® brand eggshell membrane has shown good efficacy in relieving joint pain and stiffness in several clinical trials [9-12]. Eggshell membrane is primarily composed of collagen type I [13], but also of other bioactive components, namely glycosaminoglycans including dermatan sulfate, chondroitin sulfate, hyaluronic acid and hexosamines, such as glucosamine [14,15]. These constituents have been shown to have beneficial effects in the treatment of OA [16,17]. NEM® brand eggshell membrane has been shown to down-regulate various pro-inflammatory cytokines, including interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) both in vitro [18] and in vivo [19]. ESM Technologies, LLC (Carthage, MO, USA), has developed methods to efficiently and effectively separate eggshell membrane from eggshells. The isolated membrane is then partially hydrolyzed using a proprietary process and dry-blended to produce NEM® brand eggshell membrane.

Here, we report the findings of the single-center, 2-month open-label, study that was designed to evaluate the efficacy of NEM® in the

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reduction joint pain, stiffness and functional disability in patients with moderate to severe knee OA and safety and tolerability of NEM® supplementation.

Patients and Methods

Study design

This study was performed according to a prospective, single-center, open-label design and was conducted at the Institute of Rheumatology, Belgrade, Serbia and conducted in accordance with the Declaration of Helsinki to ensure protection of human subjects.

The study protocol was reviewed and approved by an ethics committee at the study site. All OA patients enrolled in the study were appropriately informed about the study and signed informed consent.

The patients with knee OA were treated with oral NEM® 500 mg capsules (Pharmanova, Serbia) once daily for 60 days. NEM® capsules were stored in closed containers at ambient temperature. Patients were required to stop all current pain relief medications, except for paracetamol, for at least 15 days for NSAID and 3 months for glucocorticoids prior to enrollment. Clinic visits were scheduled at 10, 30, and 60 days following the onset of treatment. Treatment compliance was checked at clinic visits by patient interview and by counting the number of unused doses of the study medication. Paracetamol was allowed for pain relief, if necessary, up to 4 tablets (500mg) per day. Subjects recorded the time and amount of paracetamol taken in patient diaries.

Study population

The study included 70 patients with knee osteoarthritis. Inclusion criteria for participation in the study were: patients aged 45-75 years diagnosed with knee osteoarthritis [20] and with persistent knee pain of at least month duration and not associated with the recent trauma; the persistent knee pain associated with OA with a baseline score of 20 mm to 70 mm on the Patient's Assessment of Arthritis Pain-Visual Analog Scale (VAS) and patients that have been diagnosed with radiographic grades I-III of OA according to the Kellgren-Lawrence (KL) score [21].

Exclusion criteria for participation in the study were: pregnancy or breastfeeding, known allergy to eggs or egg products, hypersensitivity to any of the ingredients of NEM® capsules, rheumatic inflammatory diseases or systemic connective tissue diseases, co-morbidities including malignant, hematological, liver, kidney or metabolic diseases such as diabetes mellitus, body mass index greater than $\geq 29.9 \text{ kg/m}^2$, treatments with glucosamine, chondroitin sulfate, or methylsulfonylmethane (MSM), collagen, hyaluronic acid in the last three months, medications with NSAID in the last 15 days or corticosteroids in the last 3 months, and any other criteria which, by the investigator's opinion, would jeopardize patient's compliance with the study protocol.

Treatment response

The primary outcome measure of this study was to evaluate the effectiveness of NEM® in reducing pain and stiffness associated with OA of the knee. The primary endpoints were the change in Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC-overall, pain, stiffness and function), Visual Analog Scale for Pain (VAS pain) and Lequesne Algofunctional Index measured after 10, 30 and 60 days of NEM® supplementation. Patient's and physician's estimation of disease activity were also estimated.

The WOMAC OA index is a tridimensional self-administered health

status measure of pain, stiffness and physical functional disability [22]. The "pain," "stiffness" and "function" subscales consist of five, two and seventeen items, respectively. Each of the overall 24 questions is graded on a scale ranging from "none" to "extreme" with five possible answers to every question (0=none, 1=mild, 2=moderate, 3=severe, 4=extreme). The maximum score is 20 points for pain, 8 points for stiffness and 68 points for the physical function. Higher scores indicate the presence of worse symptoms, greater limitations and poorer health. Endpoints were then compared to pretreatment assessments.

A Visual Analog Scale (VAS) for pain was used to evaluate the severity of joint pain in OA patients and ranged from 0 (no problem) to 100 mm (extreme problems). VAS is considered to be reliable and valid for the assessment of subjects with specific knee conditions [23]. In addition, patient's and physician's assessment of disease activity scores were collected using a visual-analog scale.

The Lequesne OA index is a disease-specific questionnaire, which directly estimates symptoms and functions and has an interview format [24,25]. Lequesne questionnaire includes three sections with a total of 11 questions: pain or discomfort (5 items), maximum distance walked (2 items) and activities of daily living (4 items). The sum of all questions is the overall Lequesne OA index score. Each section has a score ranging from 0 to 8, resulting in a total score between 0 and 24. Higher scores indicate a worse health condition. A sum between 1 and 4 denotes a mild disability, 5-7 moderate, 8-10 severe, 11-13 very severe and greater than or equal to 14 extremely severe.

In a Global Assessment patients and physicians rated the efficacy and the safety and tolerability of NEM® on a scale ranging from: 1=no effect, 2= bad, 3=moderate, 4= good, 5=excellent following the 60 days of treatment.

Adverse events

Secondary objectives of the study were to evaluate tolerability and any adverse reactions associated with supplementation with NEM®. The subjects' self-assessment records were reviewed. Adverse events were assessed by the clinical investigator at each study visit.

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 i MedCalc Version 8.1 statistical programs. The internal consistency reliability of the WOMAC and Lequesne algofunctional indices was tested with Cronbach's alpha coefficient [26] which showed acceptable reliability ($\alpha=0.96$ for WOMAC and $\alpha=0.84$ for Lequesne indices). Descriptive statistics were performed to calculate the means, standard deviations, standard error of mean, medians, minimum and confidence interval where appropriate. For categorical variables, frequencies and percentages were provided. Following evaluation for normality, data determined to be parametric were evaluated by univariate analysis of variance (ANOVA). If ANOVA verified significance at $p < 0.05$, pairwise comparisons were made using a parametric test to identify statistical differences. Post-baseline statistical analyses were done as repeated measures univariate analysis of variance (RM-ANOVA) with post hoc Bonferroni analysis. Correlation between numerical variables has been done by Pearson coefficient (r). Statistical significance was accepted at $p < 0.05$.

Results

Demographic and clinical characteristics of OA patients

A total of seventy subjects between the ages of 45 and 75 with

osteoarthritis of the knee were enrolled in the study. The mean age of all enrolled subjects was 64.0 years. Of all enrolled patients 6% were between the ages of 45 and 50, 17% between 51 and 60, 54% between 61 and 70 and 23% between 71 and 75 years. Of these subjects, fifty-nine (84.3%) were female and eleven (15.7%) were male. The distribution of OA patients by age between genders was similar, with no significant difference in age between females and males. The mean body-mass Index (BMI) of all enrolled patients was 25.9, 23 patients (32.9%) had BMI 18.0-24.9, while 47 patients (67.1%) had BMI 25.0-29.9. The mean disease duration of all enrolled patients was 80.6 months with median duration of 48 months (minimum of 7 and maximum of 404 months). The majority of patients (76%) had disease duration in the range of zero to 120 months. Of the seventy patients, forty-two (60.0%) had bilateral incidence of knee OA. Of all patients, 16 had less severe disease (Kellgren-Lawrence score grade 1); while 54 patients had more severe disease (Kellgren-Lawrence score grade 2/3). Bilateral affection of knee joints was present in 9 patients with grade 1, in 18 patients with grade 2 and in 15 patients with grade 3 KL score. The mean disease duration was 36.2 months in patients with KL grade 1, 73.9 months in those with grade 2 and 122.5 months in grade 3 patients. All seventy subjects completed baseline assessments and the 2-month study per the protocol. Compliance with the study treatment regimen was good. Demographic and clinical characteristics of OA patients are presented in Table 1.

Effects of NEM® supplementation on WOMAC knee osteoarthritis indices

Analysis of the primary outcome measure revealed that supplementation with NEM® resulted in a significant treatment response from baseline at all-time points for overall WOMAC scores

Age (yrs), mean (SEM)		64.0 (0.9)
Gender	Male N (%)	11 (15.7)
	Female N (%)	59 (84.3)
Height (cm), mean (SEM)		167.6 (1.2)
Weight (kg), mean (SEM)		72.9 (1.3)
Body-mass Index (kg/m ²), mean (SEM)		25.9 (0.3)
Disease duration (months), mean (SEM)		80.6 (8.3)
Disease duration (months)		
0-120 (N, %)		53 (76)
120-240 (N, %)		14 (20)
240-360 (N, %)		2 (3)
360-480 (N, %)		1 (1)
Affected joints		
Left knee (N, %)		13 (18.6)
Right knee (N, %)		15 (21.4)
Bilateral (N, %)		42 (60.0)
Radiographic classification (Kellgren-Lawrence score)		
Grade 1 (N, %)		16 (22.9)
Grade 2 (N, %)		32 (45.7)
Grade 3 (N, %)		22 (31.4)

Table 1: Demographic and clinical characteristics of patients with knee OA.

Days post-treatment	Pain	Stiffness	Function	Overall
Baseline	8.1 ± 0.3 ^{a, b, c}	3.1 ± 0.2 ^{a, b, c}	30.4 ± 1.0 ^{a, b, c}	41.5 ± 1.4 ^{a, b, c}
10 days	6.5 ± 0.4 ^{a, d, e}	2.7 ± 0.2 ^{a, d, e}	25.4 ± 1.1 ^{a, d, e}	34.6 ± 1.6 ^{a, d, e}
30 days	5.5 ± 0.4 ^{b, d, f}	2.0 ± 0.2 ^{b, d, f}	20.8 ± 1.2 ^{b, d, f}	28.3 ± 1.6 ^{b, d, f}
60 days	3.9 ± 0.4 ^{c, e, f}	1.5 ± 0.2 ^{c, e, f}	16.7 ± 1.2 ^{c, e, f}	22.1 ± 1.6 ^{c, e, f}

Results are presented as mean ± SEM (n=70). p<0.05: ^abaseline vs. 10 days; ^bbaseline vs. 30 days; ^cbaseline vs. 60 days; ^d10 vs. 30 days; ^e10 vs. 60 days; ^f30 vs. 60 days; p values were determined by repeated measures univariate analysis of variance (RM-ANOVA, post hoc Bonferroni)

Table 2: WOMAC scores in NEM® supplemented patients with knee OA at baseline and after 10, 30 and 60 days of therapy.

in patients with knee OA in this study. Moreover, overall WOMAC scores significantly decreased between all-time points over the study period as shown in Table 2.

A significant decrease in overall WOMAC scores was observed after only 10 days (16.6% reduction, p=0.012) following NEM® treatment. After 30 days (31.8% reduction, p<0.0001) and 60 days (46.7% reduction, p<0.0001) of NEM® therapy further improvement of WOMAC scores was observed. Analysis of pain related WOMAC scores revealed significant reduction of pain from baseline at all-time points. Pain related WOMAC scores significantly decreased between the all-time points over the study period (Table 2). Reduction of pain was observed after only 10 days (19.8% reduction, p<0.0001), with further reductions after 30 days (32.1% reduction, p<0.0001) and 60 days (51.9% reduction, p<0.0001) of NEM® therapy.

Supplementation with NEM® resulted in significant reduction of stiffness from baseline at all-time points. Stiffness related WOMAC scores significantly decreased between all-time points over the study period (Table 2). Reduction of stiffness was observed after only 10 days (12.9% reduction, p=0.012), after 30 days (35.5% reduction, p<0.0001) and after 60 days (51.6% reduction, p<0.0001) of NEM® supplementation.

Physical function improved after supplementation with NEM® as revealed by significantly decreased function related WOMAC scores from baseline at all-time points. Namely, mean function WOMAC subscores showed a 16.4% absolute improvement at 10 days (p<0.0001), 31.6% at 30 days (p<0.0001) and 45.1% at 60 days (p<0.0001). Also, significant improvement in function was observed between all study points over the study period as shown in Table 2.

Next, we analyzed the overall WOMAC scores in NEM® supplemented patients with knee OA classified according to Kellgren-Lawrence score at baseline and 10, 30, and 60 days post-treatment as shown in Table 3. Patients with KL grade 1 had significantly lower overall WOMAC scores compared to those with KL grade 2 (p=0.018) and KL grade 3 (p=0.001) at baseline. After 30 days of NEM® supplementation overall WOMAC scores remained significantly higher in patients with KL grade 3 compared to those with KL grade 1 (p=0.002) and KL grade 2 (p=0.001). Importantly, at 60 days following NEM® supplementation there were no differences in overall WOMAC scores between patients with KL grade 1, grade 2 and grade 3.

Effects of NEM® supplementation on joint pain and overall disease activity

Assessment of joint pain using VAS revealed that supplementation with NEM® resulted in significant reduction of pain from baseline at all-time points. Moreover, pain significantly decreased on VAS between all-time points over the study period as shown in Table 4. In comparison to baseline values, significant reduction of pain was observed at 10 days (19.6% reduction, p<0.0001), at 30 days (31.8% reduction, p<0.0001) and at 60 days (49.0% reduction, p<0.0001) following NEM® supplementation. The similar rate of reduction of VAS

pain and pain related WOMAC scores was noticed from baseline at all-time point as shown in Figure 1.

Patients' evaluation of disease activity using VAS revealed significant treatment response from baseline at all-time points. Decrease of disease activity was observed after 10 days by 17.9% ($p < 0.0001$), after 30 days by 32.7% ($p < 0.0001$) and after 60 days by 46.2% ($p < 0.0001$) of NEM® therapy. A significant decrease of disease activity was observed when comparison was done between all-time points over the study period (Table 4).

Supplementation with NEM® resulted in a significant decrease in disease activity from baseline at all-time points as estimated by physicians. Decrease of disease activity was observed at 10 days by 21.6% ($p < 0.0001$), at 30 days by 40.9% ($p < 0.0001$) and by 53.1% ($p < 0.0001$) at 60 days post-treatment in comparison to baseline values.

Disease activity significantly decreased when comparisons were done between each time points over the study period (Table 4).

There was no significant difference between patient's and physician's assessment of disease activity using VAS.

Effects of NEM® supplementation on Lequesne knee osteoarthritis indices

Supplementation with NEM® resulted in a significant treatment response as reflected by significantly decreased overall Lequesne scores from baseline at all-time points. Moreover, overall Lequesne scores significantly decreased when comparisons were done between all-time points over the study period as shown in Table 5. A significant decrease in overall Lequesne scores was observed after 10 days (11.2% improvement, $p = 0.0002$), 30 days (24.0% improvement, $p < 0.0001$) and

Days post-treatment	Grade 1 (n=16)	Grade 2 (n=32)	Grade 3 (n=22)
Baseline	33.2 ± 1.7 ^{a, b}	42.4 ± 2.2 ^a	46.3 ± 2.1 ^b
10 days	31.2 ± 1.6	32.2 ± 2.5	40.6 ± 3.1
30 days	23.3 ± 1.9 ^b	24.4 ± 2.3 ^c	37.7 ± 2.8 ^{b, c}
60 days	18.8 ± 2.2	20.1 ± 2.7	27.4 ± 2.8

Results are presented as mean ± SEM (n=70), $p < 0.05$: ^agrade 1 vs. grade 2; ^bgrade 1 vs. grade 3; ^cgrade 2 vs. grade 3; p values were determined by repeated measures univariate analysis of variance (RM-ANOVA, post hoc Bonferroni)

Table 3: Overall WOMAC scores in NEM® supplemented patients with knee OA classified according to Kellgren-Lawrence score at baseline and at 10, 30 and 60 days after therapy.

Days post-treatment	VAS pain	VAS disease activity (patients)	VAS disease activity (physicians)
Baseline	53.1 ± 1.7 ^{a, b, c}	49.8 ± 1.9 ^{a, b, c}	50.1 ± 1.5 ^{a, b, c}
10 days	42.7 ± 2.2 ^{a, d, e}	40.9 ± 2.2 ^{a, d, e}	39.3 ± 2.0 ^{a, d, e}
30 days	36.2 ± 2.4 ^{b, d, f}	33.5 ± 2.2 ^{b, d, f}	29.6 ± 2.0 ^{b, d, f}
60 days	27.1 ± 2.4 ^{c, e, f}	26.8 ± 2.5 ^{c, e, f}	23.5 ± 2.2 ^{c, e, f}

Results are presented as mean ± SEM (n=70), $p < 0.05$: ^abaseline vs. 10 days; ^bbaseline vs. 30 days; ^cbaseline vs. 60 days; ^d10 vs. 30 days; ^e10 vs. 60 days; ^f30 vs. 60 days; p values were determined by repeated measures univariate analysis of variance (RM-ANOVA, post hoc Bonferroni)

Table 4: Joint pain, patient's and physician's disease activity VAS in NEM® supplemented patients with knee OA at baseline and at 10, 30, and 60 days after therapy.

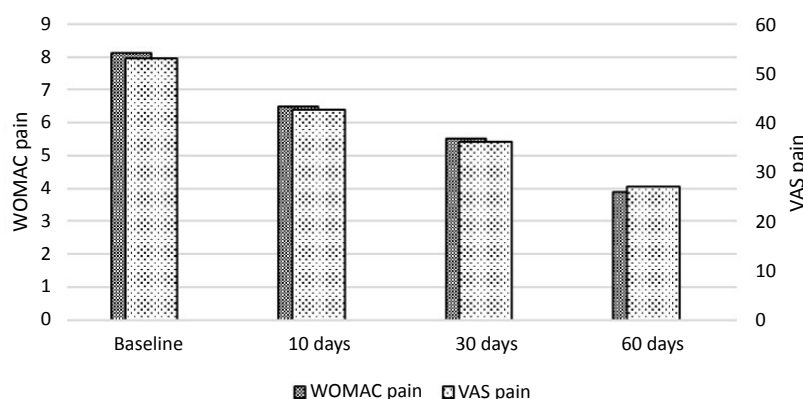


Figure 1: The similar rate of reduction of joint pain was noticed from baseline at all-time points in WOMAC pain and VAS pain in NEM® supplemented patients with knee OA.

Days post-treatment	Pain or Discomfort	Maximum Distance Walked	Activities of Daily Living	Overall
Baseline	5.1 ± 0.2 ^{a, b, c}	3.2 ± 0.2 ^{b, c}	4.2 ± 0.1 ^{a, b, c}	12.5 ± 0.4 ^{a, b, c}
10 days	4.3 ± 0.2 ^{a, d, e}	3.0 ± 0.1 ^{d, e}	3.8 ± 0.2 ^{a, d, e}	11.1 ± 0.4 ^{a, d, e}
30 days	3.8 ± 0.2 ^{b, d, f}	2.7 ± 0.1 ^{b, d}	3.1 ± 0.2 ^{b, d, f}	9.5 ± 0.5 ^{b, d, f}
60 days	3.1 ± 0.2 ^{c, e, f}	2.3 ± 0.2 ^{c, e}	2.5 ± 0.1 ^{c, e, f}	7.9 ± 0.5 ^{c, e, f}

Results are presented as mean ± SEM (n=70), $p < 0.05$: ^abaseline vs. 10 days; ^bbaseline vs. 30 days; ^cbaseline vs. 60 days; ^d10 vs. 30 days; ^e10 vs. 60 days; ^f30 vs. 60 days; p values were determined by repeated measures univariate analysis of variance (RM-ANOVA, post hoc Bonferroni)

Table 5: Lequesne scores in NEM® supplemented OA patients at baseline and at 10, 30 and 60 after therapy.

60 days (36.8% improvement, $p < 0.0001$) of NEM® therapy compared to baseline overall Lequesne scores.

Analysis of pain or discomfort related Lequesne scores revealed significant reduction of pain from baseline at all-time points. Moreover, pain related Lequesne scores significantly decreased between all-time points over the study period (Table 5). Reduction of pain was observed after 10 days (15.7% reduction, $p = 0.0002$), 30 days (25.5% reduction, $p < 0.0001$) and 60 days (39.2% reduction, $p < 0.0001$) following NEM® supplementation.

Supplementation with NEM® resulted in significantly lower distance related Lequesne scores from baseline at 30 days (15.6% improvement, $p = 0.01$) and at 60 days (28.1% improvement, $p < 0.0001$). The improvement of distance related Lequesne scores was not significant from baseline at 10 days (6.3% improvement, $p = 0.64$) (Table 5).

Post-treatment Lequesne activities of daily living related scores were significantly lower at all-time points compared to baseline values. Namely, mean Lequesne activities of daily living subscores showed a 9.5% reduction at 10 days ($p = 0.002$), 26.2% reduction at 30 days ($p < 0.0001$) and 40.5% reduction at 60 days ($p < 0.0001$).

Next, we analyzed the overall Lequesne scores in NEM® supplemented patients with knee OA classified according to Kellgren-Lawrence score at baseline and after 10, 30, and 60 days of NEM® treatment as shown in Table 6. Patients with KL grade 3 had significantly higher overall Lequesne scores compared to those with KL grade 1 ($p < 0.0001$) and KL grade 2 ($p < 0.0001$) at baseline. This difference remained at 30 days post-treatment as overall Lequesne scores were significantly higher in patients with KL grade 3 compared to those with KL grade 1 ($p < 0.0001$) and KL grade 2 ($p < 0.0001$). Of note, there were no differences in overall Lequesne scores between patients with KL grade 1, grade 2 and grade 3 after 60 days of NEM® supplementation.

Correlation analysis revealed the significant association of patients' age, BMI, and disease duration with overall Lequesne scores at baseline and at all-time points over the study period as shown in Table 7.

Global assessment of NEM® efficacy and analgesic use

In a Global Assessment, greater than 68% of patients rated

Days post-treatment	Grade 1 (n=16)	Grade 2 (n=32)	Grade 3 (n=22)
Baseline	10.4 ± 0.8 ^b	11.6 ± 0.6 ^c	15.2 ± 0.7 ^{b, c}
10 days	10.0 ± 0.7 ^b	9.8 ± 0.6 ^c	13.9 ± 0.7 ^{b, c}
30 days	8.1 ± 0.8 ^b	8.2 ± 0.5 ^c	12.5 ± 0.8 ^{b, c}
60 days	7.3 ± 0.8	7.1 ± 0.7	9.6 ± 0.7

Results are presented as mean ± SEM (n=70), $p < 0.05$: ^agrade 1 vs. grade 2; ^bgrade 1 vs. grade 3; ^cgrade 2 vs. grade 3; p values were determined by repeated measures univariate analysis of variance (RM-ANOVA, post hoc Bonferroni)

Table 6: Overall Lequesne scores in NEM® supplemented OA patients classified according to Kellgren-Lawrence score at baseline and at 10, 30 and 60 days after therapy.

	Lequesne score			
	baseline	at 10 days	at 30 days	at 60 days
BMI	0.325**	0.295*	0.321**	0.344**
OA duration	0.298*	0.245*	0.261*	N.S.
Age	N.S.	0.349**	0.361**	0.313**

Pearson correlation coefficient; * $p < 0.05$; ** $p < 0.01$, N.S.; non-significant

Table 7: Correlations between patients' age, BMI and disease duration and overall Lequesne scores at baseline and after 10, 30, and 60 days of NEM® supplementation.

the efficacy of NEM® as good or excellent following 60 days of supplementation (Table 8). Physicians also rated the treatment effective in subjects, and greater than 78% of physicians rated the efficacy of NEM® as good or excellent (Table 9).

More than 98% of patients rated the safety and tolerability of NEM® as good or excellent (Table 8) and all physicians rated the safety and tolerability of NEM® as good or excellent (Table 9). Prior to study commencement, patients consumed on average 1.50 ± 0.11 (mean ± SEM) tablets of paracetamol per day. Analgesic use had dropped considerably to 0.44 ± 0.08 ($p < 0.001$) tablets per day at 60 days of supplementation with NEM®. The use of paracetamol was not significantly reduced after 10 days, but the significant reduction was observed after 30 days (55.3%; $p < 0.001$) and 60 days (70.7%; $p < 0.001$) following the treatment with NEM®.

There were no serious adverse events reported during the study. There were three adverse events reported, one was a skin rash which was not related to allergic reaction to medication, constipation and stomach discomfort. All three reported adverse events were mild and transient and they might not be related to the study material. All three patients completed the 2-month study per the protocol.

Discussion

Osteoarthritis is a common disease and it is estimated that about one-third of the population have some form of OA in European countries [27,28]. Patients with OA experience varying degrees of chronic pain and joint stiffness which largely contributes to functional impairment and decreased quality of life [29]. It is expected that the incidence of OA will increase worldwide as population ages. Therefore, it is important that OA patients have effective and safe treatment options.

This clinical trial was designed to evaluate the efficacy and safety of NEM® as a treatment option for knee OA. Results from this study suggest that NEM®, 500 mg taken once daily, is both effective and safe for management pain associated with knee OA and considerably improves flexibility of the affected joints. NEM® has the added benefit as the use of analgesics significantly dropped over the study period. This study demonstrated that NEM® supplementation resulted in rapid

Patient's Global Assessment				
	Efficacy		Safety and Tolerability	
	Number	Frequency	Number	Frequency
Excellent	30	42.90%	62	88.60%
Good	18	25.70%	7	10.00%
Moderate	16	22.80%	1	1.40%
Bad	3	4.30%	0	0.00%
No effect	3	4.30%	0	0.00%

Table 8: Patient's Global Assessment of efficacy and safety and tolerability following 60 days of NEM® supplementation.

Physician's Global Assessment				
	Efficacy		Safety and Tolerability	
	Number	Frequency	Number	Frequency
Excellent	30	42.90%	66	94.30%
Good	25	35.70%	4	5.70%
Moderate	13	18.50%	0	0.00%
Bad	2	2.90%	0	0.00%
No effect	0	0.00%	0	0.00%

Table 9: Physician's Global Assessment of efficacy and safety and tolerability following 60 days of NEM® supplementation

(10 days) responses for WOMAC pain (19.8% reduction) and stiffness (12.9% reduction). The reduction of pain and stiffness was continuous over the study period and by the end of the follow-up period (60 days) the reduction of pain and stiffness was substantial (51.9% reduction and 51.6% reduction, respectively). By the end of the follow-up period (60 days) the mean response for function had markedly improved (45.1%). The primary outcome measures as assessed by overall WOMAC and Lequesne indices showed significant treatment effects (46.7% improvement and 36.8% improvement, respectively). Evaluation of VAS pain also revealed rapid (10 days) responses (19.6% reduction) and further reduction at 60 days (49.0%). Patient's and physician's evaluation of disease activity utilizing VAS was similar and showed significant treatment response by the end of the follow-up period (46.2% improvement and 53.1% improvement, respectively). In a Global Assessment, the majority of patients (68.6%) and physicians (78.6%) rated the efficacy of NEM® as excellent or good. According to published criteria for a response to treatment for osteoarthritis [30] treatment response to NEM® may be classified as an improvement in WOMAC pain and function as an absolute increase in the mean response rate of 35% after supplementation is considered a clinically meaningful treatment effect [10].

Our findings are in accordance with results from previous clinical studies of NEM® supplementation in patients with OA that were conducted in USA, Germany and Italy [9-12]. The treatment response rates in these studies ranged from 33% to 73% improvement of pain. In agreement with our results, in these studies the statistically significant treatment effects was observed after only 10 days following NEM® supplementation. The variations in treatment rates reported in these studies may be related to differences in OA severity and mean pain and function scores at baseline.

In line with previous studies, our findings confirm excellent safety profile for NEM® as there were no reports of adverse events or serious adverse events associated with treatment. There are no known side effects of NEM®, except from obvious egg allergy concern. This is of key importance as OA is a condition that requires long-term treatment. The common treatment option for OA is analgesics and NSAIDs which are known to lead to gastric [31] and cardiovascular [32] complications. The reduction of use of these drugs adds to the safety benefit of NEM® supplementation.

In this clinical trial no subjects withdrew from the study and there was good treatment compliance. This was open-label clinical study therefore the placebo effect cannot be excluded. We believe that this limitation is minor when considering the convincing clinical evidence for the beneficial effects of NEM® in the treatment of knee osteoarthritis.

Conclusion

The results from this open-label clinical study demonstrate that NEM® may be a viable therapeutic option for the management of pain and stiffness associated with osteoarthritis of the knee. In this clinical study, NEM®, 500 mg taken once daily, significantly reduced both pain and stiffness rapidly (10 days) and this effect continued to improve through 60 days of NEM® supplementation. This beneficial effect of NEM® was accompanied with significant reduction in the amount of analgesic consumed during the study period. NEM® supplementation has shown to be both safe and effective in managing knee osteoarthritis.

Highlights

- NEM® supplementation significantly reduced pain, stiffness and functional disability in patients with moderate and severe knee OA

- NEM® supplementation was safe and well tolerated

- NEM® supplementation was accompanied with the reduction of the amount of analgesic consumed by patients with knee OA

Acknowledgment

The study sponsor was Pharmanova, Belgrade, Serbia.

Conflict of Interest

ND, SN, MB, AKN, KV, NP received consulting fees from Pharmanova, Serbia. VK is employed by the study sponsor (Pharmanova, Serbia). The authors report no other conflicts of interest in this work.

References

1. Felson DT (2006) Osteoarthritis of the knee. *N Engl J Med* 354: 841e8.
2. Benito MJ, Veale DJ, Fitz Gerald O, Van Den Berg WB, Bresnihan B (2005) Synovial Tissue Inflammation in Early and late Osteoarthritis. *Ann Rheum Dis* 64: 1263-1267.
3. Goldring Sr, Goldring Mb (2004) The Role of Cytokines in Cartilage Matrix Degeneration in Osteoarthritis. *Clin Orthop Relat Res* 427: S27-S36.
4. Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, et al. (2005) Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. *Arthritis Rheum* 52: 128-135.
5. Altman RD (1999) Ibuprofen, acetaminophen and placebo in osteoarthritis of the knee: a six-day double-blind study. *Arthritis Rheum* 42: S403.
6. Case JP, Baliunas AJ, Block JA (2003) Lack of efficacy of acetaminophen in treating symptomatic knee osteoarthritis: A randomized, double-blind, placebo-controlled comparison trial with diclofenac sodium. *Arch Intern Med* 163: 169-178.
7. Geba GP, Weaver AL, Polis AB, Dixon ME, Schnitzer TJ, et al. (2002) Efficacy of rofecoxib, celecoxib, and acetaminophen in osteoarthritis of the knee: a randomized trial. *JAMA* 287: 64-71.
8. Towheed TE, Maxwell L, Judd MG, Catton M, Hochberg MC, et al. (2006) Acetaminophen for osteoarthritis. *Cochrane Database Syst Rev* 1: CD004257.
9. Ruff KJ, DeVore DP, Leu MD, Robinson MA (2009) Eggshell Membrane: A Possible New Natural Therapeutic for Joint and Connective Tissue Disorders. Results from Two Open-Label Human Clinical Studies. *Clin Interventions in Aging* 4: 235-240.
10. Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW (2009) Eggshell Membrane in the Treatment of Pain and Stiffness from Osteoarthritis of the Knee: A Randomized, Multicenter, Double-Blind, Placebo-Controlled Clinical Study. *Clin Rheum* 28: 907-914.
11. Danesh U, Seybold M, Rittinghausen R, Treibel W, Bitterlich N (2014) NEM® Brand Eggshell Membrane Effective in the Treatment of Pain Associated with Knee and Hip Osteoarthritis: Results from a Six-Center, Open-Label German Clinical Study. *J Arthritis* 3: 136.
12. Brunello E, Masini A (2016) NEM® Brand Eggshell Membrane Effective in the Treatment of Pain and Stiffness Associated with Osteoarthritis of the Knee in an Italian Study Population. *Int J Clin Med* 7:169-175.
13. Wong M, Hendrix MJC, von der Mark K, Little C, Stern R (1984) Collagen in the Egg Shell Membranes of the Hen. *Develop Biol* 104: 28-36.
14. Baker JR, Balch DA (1962) A Study of the Organic Material of Hen's-Egg Shell. *Biochem J* 82: 352-361.
15. Long FD, Adams RG, DeVore DP (2005) Preparation of Hyaluronic Acid from Eggshell Membrane. US Patent No. 6946551.
16. Richy F, Bruyere O, Ethgen O, Cucherat M, Henrotin Y, et al. (2003) Structural and Symptomatic Efficacy of Glucosamine and Chondroitin in Knee Osteoarthritis: A Comprehensive Meta-analysis. *Arch Intern Med* 163: 1514-1522.
17. Moreland LW (2003) Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: mechanisms of action. *Arthritis Res Ther* 5: 54-67.
18. Benson KF, Ruff KJ, Jensen GS (2012) Effects of Natural Eggshell Membrane (NEM) on Cytokine Production in Cultures of Peripheral Blood Mononuclear Cells: Increased Suppression of Tumor Necrosis Factor- α Levels after In Vitro Digestion. *J Med Food* 15: 360-368.

19. Ruff KJ, DeVore DP (2014) Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane derived product. *Mod Res Inflamm* 3: 19-25.
20. Hochberg MC, Chang RW, Dwosh I, Lindsey S, Pincus T, Wolfe F (1992) The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis. *Arthritis Rheum* 35: 498-502.
21. Kohn MD, Sassoon AA, Fernando ND (2016) Classifications in Brief Kellgren-Lawrence Classification of Osteoarthritis. *Clin Orthop Relat Res* 474: 1886-1893.
22. Bellamy N, Buchanan WW, Goldsmith CH, Campbell J, Stitt LW (1988) Validation study of WOMAC: A health status instrument for measuring clinically important patient relevant outcomes to antirheumatic drug therapy with osteoarthritis of the hip or knee. *J Rheumatol* 15: 1833-1840.
23. Flandry F, Hunt JP, Terry GC, Hughston JC (1991) Analysis of subjective knee complaints using visual analog scales. *Am J of Sports Medicine* 19: 112-118.
24. Lequesne MG, Mery C, Samson M, Gerard P (1987) Indexes of severity for osteoarthritis of the hip and knee. Validation-value in comparison with other assessment tests. *Scand J Rheumatol* 16: 85-89.
25. Lequesne MG (1997) The algofunctional indices for hip and knee osteoarthritis. *J Rheumatol* 24: 779-781.
26. Terwee CB, Bot SD, De Boer MR, Van Der Windt DA, Knol DL, et al. (2007) Quality criteria were proposed for measurement properties of health status questionnaires. *Clin Epidemiol* 60: 34-42.
27. Mannoni A, Briganti MP, Di Bari M, Ferrucci L, Costanzo S, et al. (2003) Epidemiological Profile of Symptomatic Osteoarthritis in Older Adults: A Population Based Study in Dicomano, Italy. *Ann Rheum Dis* 62: 576-578.
28. Schaap LA, Peeters GM, Dennison EM, Zambon S, Nikolaus T, et al. (2011) European Project on Osteoarthritis (EPOSA): Methodological Challenges in Harmonization of Existing Data From Five European Population-based Cohorts on Aging. *BMC Musculoskeletal Disorders* 12: 272.
29. Cook C, Pietrobon R, Hegedus E (2007) Osteoarthritis and the Impact on Quality of Life Health Indicators. *Rheum Int* 27: 315-321.
30. Pham T, Van Der Heijde D, Altman RD, Anderson JJ, Bellamy N, et al. (2004) OMERACTOARSI initiative: Osteoarthritis Research Society International set of responder criteria for osteoarthritis clinical trials revisited. *Osteoarthritis Cartilage* 12: 389-399.
31. Laine L (1996) Nonsteroidal Anti-inflammatory Drug Gastropathy. *Gastrointestinal Endoscopy. Clinics of North America* 6: 489-504.
32. Singh G, Wu O, Langhorne P, Madhok R (2006) Risk of Acute Myocardial Infarction with Nonselective Non-Steroidal Anti-Inflammatory Drugs: A Meta-Analysis. *Arthritis Res Ther* 8: R153.

Efficacy and Safety of Natural Eggshell Membrane (NEM®) in Patients with Grade 2/3 Knee Osteoarthritis: A Multi-Center, Randomized, Double-blind, Placebo-Controlled, Single-crossover Clinical Study

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Abstract

Objective: To evaluate the efficacy and safety of NEM® (natural eggshell membrane), in patients with grades 2 and 3 knee osteoarthritis (OA) having significant joint pain and stiffness, in a large, multi-center clinical trial.

Subjects and methods: This study was a randomized, double-blind, placebo-controlled, multi-center, single-crossover design. One-hundred sixty subjects (male, 32; females, 134; age ≥ 40 years) with grade 2 or 3 knee OA for 1-5 years were randomized to either NEM (n=83) 500 mg once daily or placebo (n=83) for 30 days. Osteoarthritis was evaluated using the Western Ontario and McMaster Universities OA index. NEM and placebo groups were compared at baseline, day 7, and day 30. After 30 days on placebo, the placebo group crossed over while remaining blinded and was provided with NEM (500 mg) for an additional 60 days.

Results: In NEM-treated subjects, WOMAC-stiffness was reduced at day 7 ($P=0.034$ vs. placebo), and WOMAC-total ($P=0.004$), WOMAC-pain ($P=0.023$), WOMAC-stiffness ($P=0.001$), and WOMAC-function ($P=0.001$) were reduced at day 30 (vs. placebo). The number of subjects experiencing greater decreases ($\geq 20\%$) in WOMAC-pain was significantly greater in the 90-day NEM group (48%, $P=0.022$), compared to the 60-day NEM group (30%). No serious adverse events (AE) were observed in the NEM group, and there was no significant between-group difference in the total number of AEs reported (NEM, n=8; placebo, n=15).

Conclusion: In this large, multi-center study in subjects with grade 2 and 3 knee OA, NEM reduced pain and stiffness within 7-30 days, and these clinically meaningful benefits persisted for 90 days. NEM can be considered as a safe, natural intervention for inclusion as part of a comprehensive clinical protocol in the management of knee OA.

Keywords: Arthritis; Complementary therapy; Nutraceuticals; Pain; WOMAC

Abbreviations: AE(s): Adverse Events(s); NEM: Natural Eggshell Membrane; OA: Osteoarthritis; RCT: Randomized Controlled Trial; ROM: Range of Motion; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index

Introduction

Joint and connective tissue disorders are among the most common and important chronic diseases that unfavorably influence the quality of life of those afflicted. In 2010, it was estimated that osteoarthritis (OA) and rheumatoid arthritis (RA), the two most prevalent chronic rheumatic diseases, affected 3.8% and 0.24% of the global population, respectively [1]. This equates to more than 290 million people combined worldwide.

Symptomatic knee OA including knee pain and stiffness, occurs to a greater degree in females and in individuals over the age of 50 years. The incidence of OA increases with age and 50% of those 60 years and older report having chronic knee pain [2]. A gradual increase is expected in the future prevalence of OA due to the increasing elderly population and obesity rates throughout the world. A recent study of the prevalence of symptomatic knee OA in the Izmir region in Turkey found that 20.9% of those aged 40 and over were afflicted [3].

For these reasons, there is an increasing interest in studies focusing on the treatment of OA [4]. The main goal of OA treatment is to relieve the pain and other symptoms of patients, and to enhance their functional capacities. There are a variety of prescription drugs and biologicals approved for use for OA, but these options are often associated with significant side effects and are costly. Traditional pharmacological therapies include analgesics (e.g. paracetamol, oxycodone, propoxyphene, etc.) and/or non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ibuprofen, diclofenac, celecoxib), either alone or in combination [5-7]. However, these treatments are frequently associated with adverse health concerns including cardiac

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risks [8,9], gastrointestinal problems [10,11], and addiction issues associated with long-term use of pain-relieving narcotics [12,13].

For OA (and many other conditions), natural, non-prescription interventions (i.e. integrative approaches including nutraceuticals, dietary supplements, functional foods) are preferred by many patients due to their reduced potential for side effects and generally lower cost. The most intensively investigated natural products in the context of OA are glucosamine and chondroitin sulfate [14,15]. Although less costly and having an improved side effect profile compared to prescription therapies, their overall efficacy is mild with borderline clinical significance. Clearly, there remains an unmet need for additional safe and efficacious non-prescription treatment options.

Natural eggshell membrane [NEM; commercially available in the USA as NEM®] is a non-prescription, natural source of immune-modulating bioactives [16,17]. NEM has demonstrated safety and efficacy in multiple, clinical trials in relieving joint pain and stiffness in individuals with OA [18-21]. In addition, NEM has also been reported efficacious in various animal species, including rat models of OA and RA [17,22-26]. The current study was performed to confirm the efficacy and safety of NEM in a large, multi-center trial in a new geographic population of subjects with diagnosed grades 2 and 3 OA of the knee.

Subjects and Methods

Study design

This study was a randomized, double-blind, placebo-controlled, multi-center, single-crossover study conducted in accordance with local regulations, the International Conference on Harmonization (ICH) E6 Guideline for Good Clinical Practice (GCP), and the Declaration of Helsinki at the following eight study sites: Istanbul University (Istanbul and Cerrahpaşa Schools of Medicine, (Istanbul, Turkey; sites 1 and 2, respectively); Ataturk University School of Medicine (Erzurum, Turkey; site 3); Ordu University School of Medicine (Ordu, Turkey; site 4); Adnan Menderes University School of Medicine (Aydın, Turkey; site 5); Marmara University School of Medicine, Pendik Training and Research Hospital (Istanbul, Turkey; site 6); Akdeniz University School of Medicine (Antalya, Turkey; site 7); Uludag University School of Medicine (Bursa, Turkey; site 8). Ethical approval was obtained from the respective Institutional Review Board at each study site. The study was registered at ClinicalTrials.gov (Identifier # NCT02291757). The subjects were recruited as they sought treatment at one of the participating medical centers. Written, informed consent was obtained from all participants before any study-related activities. Recruitment began in October, 2013 and was completed in May, 2015.

For the initial 30-day intervention, subjects were provided with either NEM (treatment group) or placebo. After the assessment on day 30, the placebo group was switched to NEM (single-crossover, also known as a wash-in design). At the end of 90 days, clinical evaluations were performed on two groups: one of which received NEM for 60 days (60-day treatment group, former placebo group) and the other of which received NEM for 90 days (90-day treatment group, original NEM treatment group).

Subjects

The study enrolled patients aged ≥ 40 years who were admitted to the Physical Therapy and Rehabilitation Clinics with the complaint of knee pain, had OA complaints lasting for 1-5 years, were diagnosed with knee OA according to the 2010 American College of Rheumatology and the European League against Rheumatism (ACR/EULAR) Classification

criteria, and had grade 2 or grade 3 knee OA according to the Kellgren and Lawrence classification [27].

The following were the main exclusion criteria for this study: BMI > 35 kg/m²; diagnosed inflammatory syndromes such as rheumatoid arthritis, gout, pseudogout, Paget's disease, or chronic pain syndrome; severe chronic joint pain lasting for at least 3 months with a score of ≥ 80 according to the Western Ontario and McMaster Universities Osteoarthritis (WOMAC 3.1) Index [28]; known allergy to eggs or egg products; prior enrollment in any clinical study for the treatment of joint and/or connective tissue disorders in the previous 6 months; those who received any new study product in the previous 30 days; pregnant women or breastfeeding women. Patients who agreed to participate in the study but were receiving exclusionary drugs were deemed eligible to be included in the study following a 7-day wash-out period for analgesics and NSAIDs, and a 90-day wash-out period for steroids and nutraceuticals used for the treatment of joint and connective tissue disorders (e.g. glucosamine, chondroitin, methylsulfonylmethane, etc.). Only paracetamol was allowed for pain use during the study and was provided and tracked in the same manner as treatment capsules. All other pain treatments were excluded during the study period.

Randomization

The patients were randomly assigned to either the NEM or placebo groups, and were randomized centrally, according to their registration order, using a permuted-block randomization table consisting of 4 subjects per block with a constant ratio of 1:1 among all centers. The principal investigator, co-investigators, study personnel, study participants, and statisticians were blinded to the treatment until the completion of the 90-day study.

Study intervention

Natural eggshell membrane (NEM®) is produced by mechanical separation of the eggshell membrane from the eggshell of chickens, partially hydrolyzed, dry-blended, and ground to its final particle size. NEM is primarily composed of type I collagen fibrous proteins [29] and also contains glycosaminoglycans such as dermatan sulfate and chondroitin sulfate [30,31], hexosamines such as glucosamine, hexoses and fucose [32], and a substantial amount of hyaluronic acid [31]. Other constituents of eggshell membrane include sialic acid [31], desmosine and isodesmosine [33], ovotransferrin [34], lysyl oxidase [35], and lysozyme [36]. In addition, eggshell membrane has a high potential to contain bioactive peptides (or to produce them by selective hydrolysis), as it contains a considerable amount of protein.

NEM was administered in vegetarian capsules (500 mg, once daily po). Previous studies evaluating NEM in adult subjects with osteoarthritis established that the efficacious daily dose is 500 mg [19-21]. The placebo was provided in identical vegetarian capsules containing 500 mg of a comparable but inactive substance that was identical in appearance and other qualities to the NEM capsules. The patients were instructed to ingest the study capsules with water at breakfast. Treatment compliance was evaluated at clinic visits by counting any unused capsules. Paracetamol was allowed as rescue medication and was provided as part of the study. NEM ingredient was provided by ESM Technologies, LLC (Carthage, MO USA) without cost.

Clinical assessments

In addition to the demographic characteristics of the patients, their medical histories including current medications and physical

examination findings (i.e. general health, heart rate, respiration rate, blood pressure) were also recorded. The clinical assessment of OA was performed using the Likert version of the Western Ontario and McMaster Universities Osteoarthritis Index ((WOMAC; v LK3.1: Turkish language translation) and the measurement of joint range of motion (ROM) at baseline and on days 7, 30, and 90 of treatment. The WOMAC questionnaire consists of 24 questions divided into 3 subscales, Pain (5 questions, 0-20 total points), Stiffness (2 questions, 0-8 total points), and Function (17 questions, 0-68 points). The WOMAC sub-scores were summed to produce the WOMAC-total score (0-96 points). A lower score on any WOMAC scale denotes a better outcome. The patients were also questioned at each clinic visit about any adverse events that they may have had. All clinic assessments were performed a minimum of 24 hours following the most recent paracetamol dose, if applicable. The NEM and placebo groups were compared in terms of the findings on days 7 and 30. In the evaluations performed on day 90, the 60-day NEM treatment group was compared with the 90-day NEM treatment group.

Sample size estimation, statistical analyses and outcome measures

The primary end point was the difference between the NEM group vs. placebo group in the WOMAC-total score, assessed on day 30. To

detect a 15% treatment effect (vs. placebo), we estimated that a sample size of 156 patients would be required to provide a statistical power of 80%, assuming a response rate of 20% in the treatment group and response rate of 5% in the placebo group, with a 5% dropout rate. Data analyses were performed using the IBM SPSS Statistics for Windows version 22.0 (IBM Corp, Armonk, NY USA). Descriptive statistics were expressed as a number and percentage for categorical variables, and as mean \pm standard deviation (SD) for numerical variables. The Wilcoxon signed-rank test was used for normally distributed two group comparisons, whereas the Mann-Whitney U test was performed for two group comparisons for non-normally distributed variables. A P value of < 0.05 was considered statistically significant. To minimize missing data points due to dropouts for statistical calculations, the last observation carried forward (LOCF) approach was used for subjects for which at least one evaluation following the baseline visit was conducted.

Results

The trial enrollment flow diagram shows the assignment and progress of subjects during the study (Figure 1). A total of 208 candidates were assessed for eligibility by the 8 clinical sites, and 42 candidates were excluded. One-hundred-sixty-six (166) individuals qualified for randomization, with 83 assigned to the NEM group and 83 assigned to the placebo group. The distribution of the enrolled

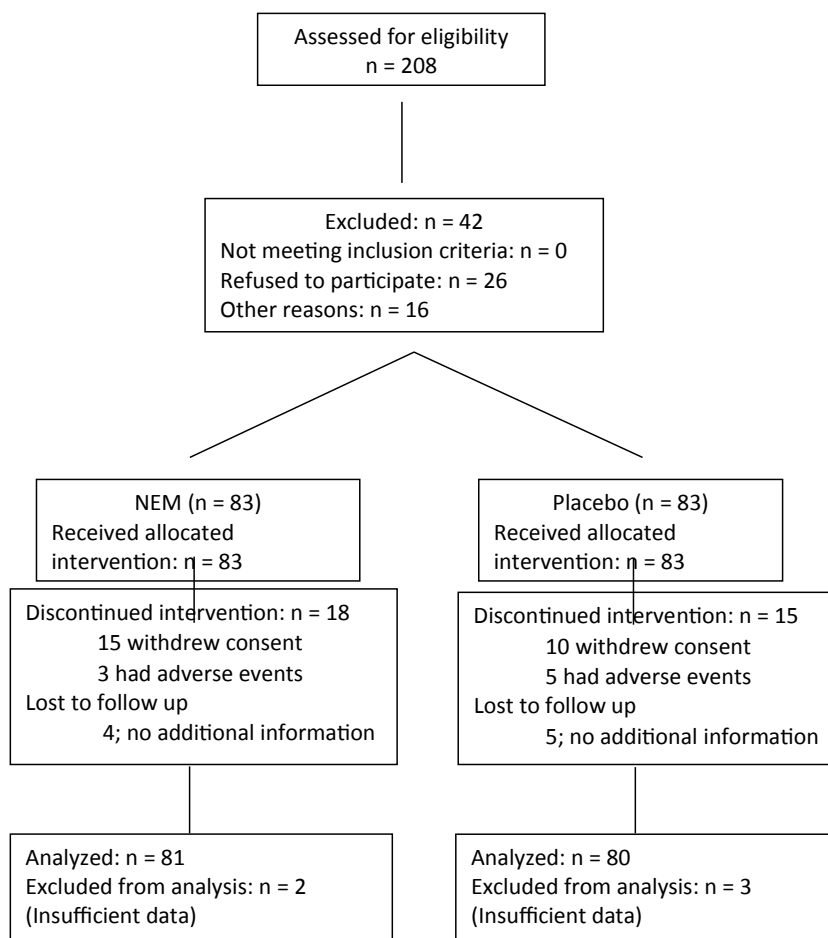


Figure 1: Trial subject enrollment flow diagram. The dates for the initiation of recruitment through the completion of this trial were October, 2013 through May, 2015, respectively.

subjects among the study sites was as follows: 16 (10%) were from site 1, 31 (19%) were from site 2, 22 (13%) were from site 3, 11 (7%) were from site 4, 16 (10%) were from site 5, 11 (7%) were from site 6, 22 (13%) were from site 7, 37 (22%) were from site 8. No serious adverse events were observed in the NEM treatment group, and there was no significant between-group difference in the number of adverse events reported (NEM, n=8; placebo, n=15). Thirty-four of the original 166 enrolled subjects dropped out during the study for unanticipated personal reasons (NEM, n=19; placebo, n=15), and 9 subjects were lost to follow-up (NEM, n=4; placebo, n=5). Table 1 shows the baseline demographic data for the enrolled subjects and indicates that both groups were statistically similar.

All clinical indices of OA were similar between the 2 groups at baseline (Table 2). The WOMAC-stiffness score at the end of the 7-day treatment period in the NEM group improved by approximately 24% from baseline (3.4 ± 1.7 ; within group $P=0.004$) and was significantly lower compared to the placebo group (NEM 2.6 ± 1.8 ; placebo 3.4 ± 2.0 ; $P=0.034$). Similarly, the WOMAC-pain score at the end of the 7-day treatment period in the NEM group improved by approximately 22% from baseline (10.1 ± 4.1 ; within group $P=0.001$). No between-group differences were observed in this or the other clinical indices.

After 30 days, WOMAC-pain and WOMAC-stiffness in the NEM group had improved from baseline by 33% and 35%, respectively (within group P both <0.001). All WOMAC-based indices, including the primary outcome measure (WOMAC-total) were significantly lower in the NEM group compared to placebo: WOMAC-total (Absolute Treatment Effect 14.9%, $P=0.004$); WOMAC-pain (Absolute Treatment Effect 12.3%, $P=0.023$); WOMAC-stiffness (Absolute Treatment Effect 18.2%, $P=0.001$); WOMAC-function (Absolute Treatment Effect 15.2%, $P=0.001$) (Table 2). No between-group differences were observed for range of motion (angles of flexion or extension).

After 90 days, final clinical assessments were performed on the original NEM group (90-day NEM) and the original placebo group (60-

Parameter	NEM (n=83)	Placebo (n=83)	P
Age, years	55.9 \pm 11.9	58.5 \pm 9.7	0.156
Gender			
Male (%)	12 (14.5)	20 (24.1)	0.168
Female (%)	71 (85.5)	63 (75.9)	
Race			
Caucasian (%)	83 (100)	83 (100)	-
Weight (kg)	76.9 \pm 11.4	78.0 \pm 11.2	0.531
Height (m)	1.6 \pm 0.1	1.6 \pm 0.1	0.734
BMI, kg/m ²	29.4 \pm 3.7	29.5 \pm 3.3	0.668
Smoking			
Present (%)	30 (36.1)	32 (38.6)	0.873
Absent (%)	53 (63.9)	51 (61.4)	
Alcohol consumption			
Present (%)	1 (1.2)	1 (1.2)	1
Absent (%)	81 (98.8)	81 (98.8)	
Blood Pressure (mm Hg) systolic /	127.1 \pm 12.4 /	127.4 \pm 16.4 /	0.133
Diastolic	80.9 \pm 14.3	79.9 \pm 8.9	0.782
Heart Rate (beats per minute)	78.3 \pm 7.7	80.2 \pm 8.3	0.135
Respirations (breaths per minute)	16.5 \pm 3.3	16.4 \pm 2.9	0.919
Oral Temperature (°C)	36.7 \pm 0.6	36.7 \pm 0.5	0.398

Data are presented as the actual number (% total number) or mean \pm standard deviation, where appropriate. Abbreviations: BMI: Body Mass Index; calculated as weight in kilograms divided by the square of height in meters

Table 1: Baseline demographic characteristics of enrolled subjects.

Time Index	NEM	Placebo	P
Baseline	(n=83)	(n=83)	
WOMAC-total	42.4 \pm 20.0	47.7 \pm 23.9	0.123
WOMAC-pain	10.1 \pm 4.1	10.8 \pm 5.2	0.551
WOMAC-stiffness	3.4 \pm 1.7	4.1 \pm 2.0	0.105
WOMAC-function	28.9 \pm 14.2	32.8 \pm 16.7	0.107
Angle of flexion	127.2 \pm 12.1	125.1 \pm 14.1	0.481
Angle of extension	0.2 \pm 3.7	2.2 \pm 21.7	0.643
Day 30	(n=81)	(n=80)	
WOMAC-total	32.8 \pm 18.7	44.0 \pm 22.8	0.004
WOMAC-pain	6.8 \pm 4.0	8.6 \pm 5.0	0.023
WOMAC-stiffness	2.2 \pm 1.7	3.4 \pm 2.1	0.001
WOMAC-function	23.8 \pm 13.0	32.0 \pm 15.7	0.001
Angle of flexion	128.7 \pm 10.7	126.6 \pm 12.7	0.529
Angle of extension	2.5 \pm 22.6	2.7 \pm 22.6	0.811

Data are presented as mean \pm standard deviation. Abbreviations: WOMAC: The Western Ontario and McMaster Universities Osteoarthritis Index.

Table 2: Clinical Indices of OA in NEM and Placebo Groups at Baseline and Day 30.

day NEM). Addition of NEM to the original placebo group resulted in a marked clinical improvement, as judged by the lack of between-group statistical significance in the WOMAC-total, WOMAC-pain, and WOMAC-stiffness scores ($P=0.193$, $P=0.140$, $P=0.079$, respectively). This difference was due to improved WOMAC scores in the original placebo group, and not due to any apparent reduction in efficacy in the original NEM group. The difference in WOMAC-function score remained statistically different between the original NEM group and the original placebo group ($P=0.002$). No between-group differences were observed for the range of motion.

A responder analysis was performed in the two groups. Interestingly, the number of patients having at least a 15% decrease in WOMAC-pain score was greater in the 90-day NEM group (71% of subjects) compared to the 60-day NEM group (53% of subjects; $P=0.025$). Similarly, the number of patients having at least a 20% decrease in WOMAC-stiffness score was greater in the 90-day NEM group (48% of subjects) compared to the 60-day NEM group (30% of subjects; $P=0.022$).

Safety and tolerability

Overall, the treatment was well tolerated by the patients, with no between-group statistical difference in adverse events. There was a total of 8 (9.6%) adverse events (AEs) reported in the NEM group, and none were deemed serious by study investigators. Three AEs (i.e. rash, nausea) were judged to be related to the study material, perhaps due to undiagnosed egg allergy. There were a total of 15 (18.1%) AEs reported in the placebo group; 3 of these were serious AEs. Three AEs in the placebo group were believed to be related to the study material. Rescue medication (paracetamol) use was comparable (~50.0% utilization rate) between the two groups. Treatment compliance was excellent, as judged by approximately 92% of the original NEM group and 88% of the original placebo group returning fewer than 10 of the allocated capsules.

Discussion

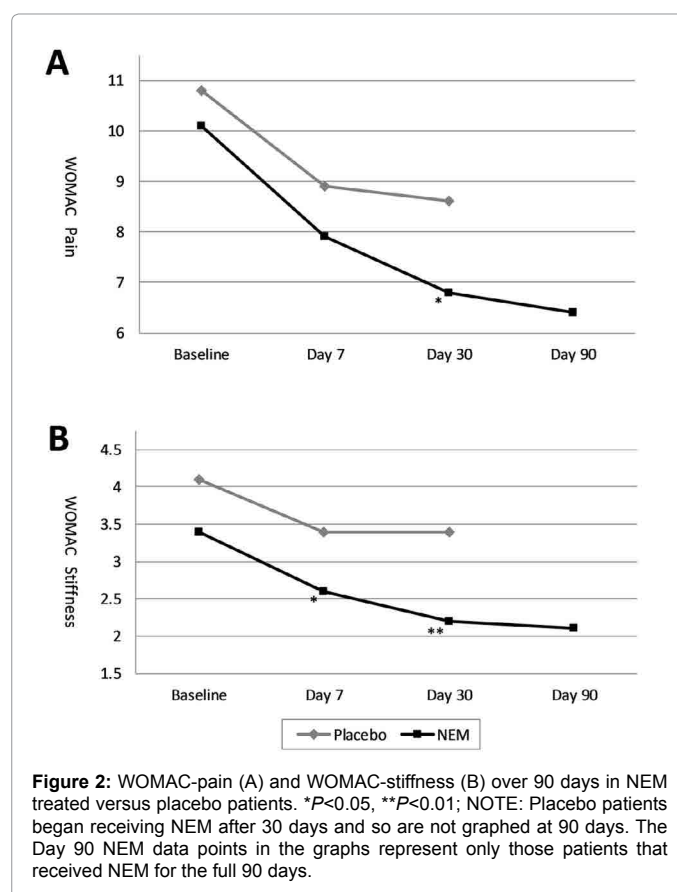
NEM was used at a dose of 500 mg/day to assess its efficacy and safety in patients with grade 2 and grade 3 knee OA. The principal finding of this study was the rapid (7 days) and persistent (through day 90) clinically meaningful improvement in validated indices (WOMAC scores) of OA, in subjects with moderate-to-severe OA of the knee who

were taking NEM (compared to placebo). Specifically, in the NEM group, the WOMAC-stiffness score was significantly reduced at day 7 and, by day 30, all major WOMAC indices (total, pain, stiffness, and function) including the primary outcome measure (WOMAC-total) were significantly improved. As has been reported previously [19], continuation on the NEM regimen increases the number of responders along with the overall magnitude of the clinical improvement. In this study, the percentage subjects experiencing greater percent decreases in the WOMAC-pain score was significantly greater in the 90-day NEM group compared to those in the 60-day NEM group. Thus, there appears to be a positive correlation between the duration of exposure to NEM, the number of responders, and the overall magnitude of effect.

Despite a significant within-group improvement at 7 days in the NEM treatment group for WOMAC-pain (-22% from baseline), there was no difference when compared to placebo. There have been a number of prior open-label clinical studies evaluating NEM in subjects with various joint and connective tissue disorders: two in the United States (U.S.) (n=11; n=28) [18], one in Germany (n=44) [20], and one in Italy (n=25) [21]. There has also been an RCT evaluating knee OA in the U.S. (n=67) [19]. These prior studies reported significant clinical improvements within 7-10 days with regard to reducing joint pain, ranging from 15.9% to 40.6%. Although the present study had a similar treatment effect size, rapid results may have been obscured by the greater severity of knee OA in our study. This is supported by the fact that WOMAC-stiffness had a similarly sized within-group treatment effect (-24% from baseline) that was also significantly different from placebo ($P=0.034$). It is mechanistically consistent that stiffness would be affected earlier than pain, as the swelling from localized inflammation is reduced. The prostaglandins that are involved in pain sensation are produced as a result of inflammation and so would take more time to resolve once inflammation diminishes. So it may be that WOMAC-pain would have reached statistical significance by 10 days as was seen in a number of the previous clinical trials mentioned above.

At the end of the placebo-controlled portion of the trial (Day 30), there was a marked difference in improvement in pain and stiffness, two symptoms of OA critically important to treat. NEM improved WOMAC-pain and WOMAC-stiffness by absolute treatment effects of 12.3% and 18.2%, respectively. These results are very consistent with 30-day absolute treatment effects found in a prior randomized controlled trial (RCT) of 67 subjects conducted in the United States (pain 10.3%; stiffness 16.8%) [19]. Our results from this much larger, multi-center study now confirm the results found previously with NEM, despite the fact that we included patients with moderate to severe knee OA. Treatment options are limited for Grade 2/3 OA, so the results presented here for a natural, non-prescription intervention like NEM are quite remarkable.

Comparison of the subjects receiving NEM for 60 versus 90 days revealed a number of noteworthy items. Of greatest importance is that NEM continued to improve WOMAC-pain and WOMAC-stiffness in the group that received NEM continuously for 90 days (Figure 2A and 2B), albeit at a reduced rate of improvement compared to earlier in the trial. The majority of symptomatic (pain & stiffness) improvement appeared to occur within the first 30 days of treatment; however, symptoms continued to improve through 90 days of treatment. This is the first RCT to evaluate NEM for this length of time and it would appear that maximal efficacy for NEM is reached around 3 months of use. Secondly, the crossover of the placebo group to NEM treatment after 30 days served as an internal check on the validity of NEM's efficacy beyond that of the placebo effect. That is, the fact that there



was a statistically significant difference between subjects taking NEM for 60 days versus those taking NEM for 90 days supports that the improvements from NEM are real, as surely the placebo effect would have diminished substantially if not completely after 4 weeks in patients with moderate to severe OA.

No improvement in either flexion or extension range of motion evaluation was observed in this study. Within the context of significant reductions in both pain and stiffness, it is reasonable to have expected a concomitant improvement in joint flexibility. Yet this was not the case. This might be attributable to the more severe OA burden in these study patients, the evaluation of only the knee in this study vs. other joints in the previous open-label study [18], or possibly due to a difference(s) in how range of motion was measured in the current vs. previous studies.

As has been reported in previous clinical studies [18-21], NEM was safe and well tolerated in the current study with no occurrence of serious adverse events or any observed difference in total number of between-group AEs. This confirms in humans what had previously been reported through *in vitro* and *in vivo* toxicity studies [37]. From a regulatory perspective in the U.S., NEM is generally recognized as safe (GRAS), with an allowable daily intake of up to 14 grams, enabling its inclusion in multiple delivery formats for foods, beverages, and dietary supplements.

The overall drop-out rate (25.3%) was greater than estimated (5%) in the sample size calculation. However, trial recruitment (166) exceeded the calculated sample size (156) by 6% and the estimated net treatment effect (15%) used in the sample size calculation was similar to the actual net treatment effect for WOMAC-pain (12%) and was

exceeded for WOMAC-stiffness (18%). These facts likely helped to mitigate the increased dropout rate and may partially explain why a treatment effect for WOMAC-stiffness was able to be detected at just 7 days. Dropouts were evenly distributed between the NEM group (n=22; 26%) and the placebo group (n=20; 24%) with no obvious differences in the reason for dropping out. Many of the patients had to travel a fair distance to the regional medical centers to participate in the study and there were 6 clinical visits, so this may have contributed appreciably to the increased drop-out rate.

The present study had a number of strengths and limitations. Major strengths of the study include the use of a large number of subjects (n=83 per group) with well-characterized OA of the knee, thus affording the appropriate statistical power. The use of a placebo group for the initial 30-day evaluation period along with the utilization of 8 individual study centers substantially minimized the possibility for experimental bias in evaluating the potential clinical benefit for NEM. Another strength of this study was the use of a well-validated clinical index of OA, namely the WOMAC index [38]. There are over 200 citations (primary studies, reviews, etc.) reporting the successful use of this self-reported health questionnaire in multiple clinical settings including OA. The major limitations of this study were the failure to include a third arm of the study evaluating a reference intervention (e.g. standard of care) for comparison, or any serum /urinary biomarker(s) of cartilage metabolism. However, these added features of the study were beyond the scope of this particular study, which was simply to evaluate NEM in a well-defined clinical population, using a large sample size spread across multiple study centers.

Conclusions

This is now the sixth clinical trial involving NEM and the largest trial to date. The therapeutic benefits reported in each of these geographically-diverse trials, including the present study, have been consistent and reproducible. Taken together, the use of NEM in the context of OA consistently yields statistically significant and clinically meaningful results. The combination of quick symptom relief (7 days) coupled with continuing long-term relief (90 days) is impressive from a food-based ingredient, and should be clinically beneficial for those suffering from OA. NEM can be considered as a safe, cost-effective, natural intervention for inclusion as part of comprehensive clinical protocol in the management of patients with knee OA, even in patients with more severe grade 2 and 3 OA.

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References

- Cross M, Smith E, Hoy D, Carmona L, Wolfe F, et al. (2014) The global burden of rheumatoid arthritis: estimates from the global burden of disease 2010 study. *Ann Rheum Dis* 73: 1316-1322.
- Busija L, Bridgett L, Williams SR, Osborne RH, Buchbinder R, et al. (2010) Osteoarthritis. *Best Pract Res Clin Rheumatol* 24: 757-768.
- Yesil H, Hepguler S, Ozturk C, Capaci K, Yesil M (2013) Prevalence of symptomatic knee, hand and hip osteoarthritis among individuals 40 years or older: a study conducted in Izmir city. *Acta Orthop Traumatol Turc* 47: 231-235.
- Fibel KH, Hillstrom HJ, Halpern BC (2015) State-of-the-art management of knee osteoarthritis. *World J Clin Cases* 3: 89-101.
- Geba GP, Weaver AL, Polis AB, Dixon ME, Schnitzer TJ (2002) Efficacy of rofecoxib, celecoxib, and acetaminophen in osteoarthritis of the knee: a randomized trial. *J Amer Med Assoc* 287: 64-71.
- Case JP, Baliunas AJ, Block JA (2003) Lack of efficacy of acetaminophen in treating symptomatic knee osteoarthritis: a randomized, double-blind, placebo-controlled comparison trial with diclofenac sodium. *Arch Intern Med* 163: 169-178.
- Towheed TE, Maxwell L, Judd MG, Catton M, Hochberg MC, et al. (2006) Acetaminophen for osteoarthritis. *Cochrane Database Syst Rev* CD004257.
- Singh G, Wu O, Langhorne P, Madhok R (2006) Risk of acute myocardial infarction with nonselective non-steroidal anti-inflammatory drugs: a meta-analysis. *Arthritis Res Ther* 8: R153.
- Solomon SD, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, et al. (2005) Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 352: 1071-1080.
- Deeks JJ, Smith LA, Bradley MD (2002) Efficacy, tolerability, and upper gastrointestinal safety of celecoxib for treatment of osteoarthritis and rheumatoid arthritis: systematic review of randomised controlled trials. *Brit Med J* 325: 619.
- Laine L (1996) Nonsteroidal anti-inflammatory drug gastropathy. *Gastrointest Endosc Clin N Am* 6: 489-504.
- Weaver M, Schnoll S (2007) Addiction issues in prescribing opioids for chronic nonmalignant pain. *J Addict Med* 1: 2-10.
- Juurink DN, Dhalla IA (2012) Dependence and addiction during chronic opioid therapy. *J Med Toxicol* 8: 393-399.
- Bruyere O, Reginster JY (2007) Glucosamine and chondroitin sulfate as therapeutic agents for knee and hip osteoarthritis. *Drugs Aging* 24: 573-580.
- Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, et al. (2006) Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. *N Engl J Med* 354: 795-808.
- Ruff KJ, Durham PL, O'Reilly A, Long FD (2015) Eggshell membrane hydrolyzates activate NF-kappaB in vitro: possible implications for in vivo efficacy. *J Inflamm Res* 8: 49-57.
- Ruff KJ, DeVore DP (2014) Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Mod Res Inflamm* 3: 19-25.
- Ruff KJ, DeVore DP, Leu MD, Robinson MA (2009) Eggshell membrane: a possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin Interv Aging* 4: 235-240.
- Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW (2009) Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin Rheumatol* 28: 907-914.
- Danesch U, Seybold M, Rittinghausen R, Triebel W, Bitterlich N (2014) NEM® brand eggshell membrane effective in the treatment of pain associated with knee and hip osteoarthritis: results from a six center, open label German clinical study. *J Arthritis* 3: 1000136-1000141.
- Brunello E, Masini A (2016) NEM® brand eggshell membrane effective in the treatment of pain and stiffness associated with osteoarthritis of the knee in an Italian study population. *Int J Clin Med* 7: 169-175.
- Wedekind KJ, Ruff KJ, Atwell CA, Evans JL, Bendele AM (2017) Beneficial effects of natural eggshell membrane (NEM) on multiple indices of arthritis in collagen-induced arthritic rats. *Mod Rheumatol* 27: 838-848.
- Wedekind KJ, Coverdale JA, Hampton TR, Atwell CA, Sorbet RH, et al. (2015) Efficacy of an equine joint supplement, and the synergistic effect of its active ingredients (chelated trace minerals and natural eggshell membrane), as demonstrated in equine, swine, and an osteoarthritis rat model. *Open Access Anim Physiol* 7: 1-15.
- Dierenfeld ES, Baum D, Hampe L, Jensen J, Atwell C, et al. (2014) Evaluation of a nutraceutical joint supplement in camels (*Camelus species*). *Amer Holst Veterin Med Assoc J* 36: 59-66.
- Bauer KL, Dierenfeld ES, Hartup BK (2014) Evaluation of a nutraceutical joint supplement in cranes. *Proc North Am Crane Workshop* 12: 27-32.
- Sim BY, Bak JW, Lee HJ, Jun JA, Choi HJ, et al. (2015) Effects of natural

- eggshell membrane (NEM) on monosodium iodoacetate-induced arthritis in rats. *J Nutr Health* 48: 310-318.
27. Kohn MD, Sassoon AA, Fernando ND (2016) Classifications in brief: Kellgren-Lawrence classification of osteoarthritis. *Clin Orthop Relat Res* 474: 1886-1893.
 28. Bellamy N (2002) WOMAC: a 20-year experiential review of a patient-centered self-reported health status questionnaire. *J Rheumatol* 29: 2473-2476.
 29. Wong M, Hendrix MJ, von der MK, Little C, Stern R (1984) Collagen in the egg shell membranes of the hen. *Dev Biol* 104: 28-36.
 30. Baker JR, BALCH DA (1962). A study of the organic material of hen's-egg shell. *Biochem J* 82: 352-361.
 31. Nakano T, Ikawa NI, Ozimek L (2003) Chemical composition of chicken eggshell and shell membranes. *Poult Sci* 82: 510-514.
 32. Picard J, Paul-Gardais A, Vedel M (1973) Sulfated glycoproteins from egg shell membranes and hen oviduct. Isolation and characterization of sulfated glycopeptides. *Biochim Biophys Acta* 320: 427-441.
 33. Starcher BC, King GS (1980) The presence of desmosine and isodesmosine in eggshell membrane protein. *Connect Tissue Res* 8: 53-55.
 34. Gautron J, Hincke MT, Panheleux M, Garcia-Ruiz JM, Boldicke T, et al. (2001) Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. *Connect Tissue Res* 42: 255-267.
 35. Akagawa M, Wako Y, Suyama K (1999) Lysyl oxidase coupled with catalase in egg shell membrane. *Biochim Biophys Acta* 1434: 151-160.
 36. Hincke MT, Gautron J, Panheleux M, Garcia-Ruiz J, McKee MD, et al. (2000) Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. *Matrix Biol* 19: 443-453.
 37. Ruff KJ, Endres JR, Clewell AE, Szabo JR, Schauss AG (2012) Safety evaluation of a natural eggshell membrane-derived product. *Food Chem Toxicol* 50: 604-611.
 38. Benson KF, Ruff KJ, Jensen GS (2012) Effects of natural eggshell membrane (NEM) on cytokine production in cultures of peripheral blood mononuclear cells: increased suppression of tumor necrosis factor-alpha levels after in vitro digestion. *J Med Food* 15: 360-368.

Development of a Novel Clinical Trial Design to Evaluate the Effects of Joint Therapeutics on Cartilage Turnover in Healthy Subjects

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Abstract

Background: Articular joint diseases such as Osteoarthritis (OA) and Rheumatoid Arthritis (RA) are quite prevalent throughout the world, particularly in adults 60 years of age and older, and result in significant costs (both financial and quality-of-life) for those that are afflicted. Owing to the lack of drugs that can halt the progression of arthritis there is an obvious current need for additional joint therapeutics. Various biomarkers have been historically evaluated to better guide the development of new therapeutic interventions. Of these biomarkers, c-terminal Cross-Linked Telopeptide of type-II collagen (CTX-II), a marker of cartilage degradation, has shown the most potential. The purpose of this investigation was to develop a clinical trial design to aid in the evaluation of chondroprotective joint therapeutics by taking advantage of the apparent sensitivity of the articular cartilage of healthy, post-menopausal women to increased exercise-induced joint strain.

Methods: Variables such as the timing of urinary CTX-II clearance and the sensitivity of the CTX-II assay were initially investigated. The sensitivity of CTX-II production to differing levels of exercise strain was then investigated in a non-interventional clinical phase in developing the trial design. The joint therapeutic Natural Eggshell Membrane (NEM[®]) was then evaluated in a subsequent open-label clinical phase as a proof of concept.

Results: There appears to be a reproducible initial increase in uCTX-II clearance within the first 2-4 hours following exercise, followed by somewhat of a plateau, with the maximum (or near maximum) level being observed 24 hours post-exercise in the 2nd void of the morning. The CTX-II assay is sensitive enough to measure changes resulting from exercise in obese and OA subjects with a 20.4% to 43.6% increase from resting. There was an obvious trend in the non-interventional clinical phase for the strenuous nature of the exercise (lifting weight>seated step machine>inclined treadmill) to affect the magnitude of the cartilage turnover of the study subjects. NEM[®] prevented exercise-induced cartilage turnover in the open-label clinical phase indicating that it is chondroprotective.

Conclusion: This trial design shows great potential to evaluate chondroprotective joint therapeutics including symptomology (i.e. joint pain and stiffness) in healthy individuals, where sparing cartilage may prevent patients from ultimately developing arthritis. By extension, this design may also enable the evaluation of chondroprotective joint therapeutics in an OA population, particularly where cartilage preservation has reached a critical stage.

Keywords: Chondroprotective; High-impact; CTX-II biomarker; Arthritis; Trial design

Introduction

Estimates by the World Health Organization show that a considerable percentage of the global population is afflicted with articular joint disease [1]. There are currently no approved Disease Modifying Osteoarthritis Drugs (DMOADs) [2] and only a handful of Disease Modifying Anti-Rheumatic Drugs (DMARDs) are approved. Both non-biologic and biologic DMARD treatment presently comes with substantial risks, either due to hepatotoxicity or increased occurrences of pathogenic infections and malignancies [3]. The lack of drugs that can safely halt the progression of arthritis (be disease modifying) combined with the fact that it is frequently diagnosed a decade or more into the disease when severity is considerably greater, results in a substantial burden for global healthcare systems. Because so many people develop these costly and debilitating diseases there is

an obvious current need for safe and effective joint therapeutics and the future need will be substantially greater. Therefore, the ability to evaluate new joint therapeutics is paramount to the approval of new molecular entities or for new indications for existing drugs to meet this need.

Various biomarkers have been evaluated through the years in an attempt to better understand arthritis progression and/or prognosis and to better guide the development of therapeutic interventions. Researchers have looked at immune cell patterns in the joints [4], serological parameters (cholesterol & triglycerides) and markers of oxidative stress (malondialdehyde & C-reactive protein) [5], synovial fluid cytokine levels (TNF- α , IL-1 β , IL-6, etc.) [6], as well as cartilage components in synovial fluid (chondroitin sulfate, glycosaminoglycans, hyaluronic acid, etc.) [7]. Many of these biomarkers suffer from a number of drawbacks, from lack of specificity (e.g. cholesterol) to difficulty in obtaining samples (e.g. synovial fluid). Because of the plethora of biomarkers from which to choose to

evaluate arthritis, the Osteoarthritis Biomarkers Network funded by the National Institutes of Health/National Institute of Arthritis, Musculoskeletal, and Skin Disease (NIH/NIAMS) proposed a classification scheme for biomarkers to provide a common format for communication of research in this area. This scheme is termed BIPED which is an acronym for Burden of disease, Investigative, Prognostic, Efficacy of intervention, and diagnostic [8]. These characteristics help to rank biomarkers as to their clinical utility in diagnosing and treating arthritis. Based upon these criteria, indicators of cartilage turnover (i.e. synthesis and degradation) have moved to the top of the list of biomarker candidates likely to be most useful. We chose to investigate c-terminal cross-linked telopeptide of type-II collagen (CTX-II), a marker of cartilage degradation, as an indicator of chondroprotective effects from joint therapeutics. Urinary CTX-II levels are known to be substantially elevated in those afflicted with articular joint diseases, but levels are also known to be elevated in a variety of healthy subsets of the population, as well. Urinary CTX-II levels have been shown to be elevated due to high-impact exercise in healthy college-aged endurance athletes such as cross-country runners by about 85% over age- and weight-matched controls but were not significantly elevated in lower-impact endurance athletes like swimmers and rowers [9]. Urinary CTX-II has also been shown to be about 2-fold higher in post-menopausal women versus age-matched pre-menopausal women and moderately elevated (~25%) in overweight people (BMI ≥ 25 kg/m²) versus normal-weight controls (BMI <25 kg/m²) [10].

The fact that large portions of the population develop arthritis combined with the fact that there are currently no approved disease-modifying or chondroprotective agents results in an obvious urgent need for safe and effective joint therapeutics. To our knowledge, there are presently no clinical models designed to evaluate chondroprotective joint therapeutics. The aim of this research investigation, therefore, was to develop a simple and rapid clinical trial design to aid in the evaluation of chondroprotective joint therapeutics, and we hoped to take advantage of the apparent sensitivity of the articular cartilage of post-menopausal women and/or overweight individuals to evaluate joint therapeutics through increasing joint strain *via* exercise while monitoring uCTX-II output.

Patients and Methods

Initial investigation of design variables

The initial critical variable investigated was to determine whether or not CTX-II production and urinary clearance would occur in a narrow enough time frame to be useful for our envisaged purpose. Individuals clear proteins at differing rates, so it was also important to determine if the clearance rate was sufficiently consistent between individual subjects to be able to obtain samples at a single point in time. There was also concern as to whether the Enzyme-Linked Immunosorbent Assay (ELISA) would be sensitive enough to measure a change in urinary CTX-II induced by exercise. That is, would the increase in uCTX-II be sufficiently large so that it would not be obscured by the variation inherent in the assays, which have generally been reported to be around 10%-15%.

To help normalize uCTX-II clearance rates, the ratio of urinary CTX-II expressed in micrograms per liter (μ g/L) to urinary Creatinine (Cr) expressed in millimoles per liter (mmol/L) was calculated and results were reported as nanograms of CTX-II per millimole of Creatinine (ng/mmol Cr). Urinary CTX-II levels were evaluated using a commercial ELISA kit from Immunodiagnosics Systems, Inc. (Urine

CartiLaps® EIA) according to manufacturer instructions. Baseline urine samples were collected from the 2nd void of the morning and were frozen (-20°C) immediately and held until needed for assaying. Thawed samples were subdivided into aliquots to avoid subsequent repeated freeze/thaw cycles that might result in aberrant repeat assay values.

Two females (ages 34 & 37) and one male (age 34) having healthy knee joints (no resting knee pain or stiffness) and one female (age 60) with diagnosed OA of the right knee all with a BMI <25 kg/m² provided urine samples for basal CTX-II level determination. The subjects subsequently performed exercises (females: 300 stairs per leg over 10-15 minutes; male: jogged 4 miles over ~45 minutes) that would be expected to increase levels of excreted CTX-II. Subjects provided subsequent urine samples approximately every 2-4 hours (for ~12 hours) and again at 24 hours to follow CTX-II clearance temporally to determine when the maximum post-exercise uCTX-II level would be observed.

These initial subjects were all regular exercisers, so we next wanted to evaluate more moderate forms of exercise in non-exercising or infrequently exercising individuals. A post-menopausal female (age 67) with healthy knee joints who did not exercise regularly provided a urine sample for basal CTX-II level determination. The subject subsequently performed exercise for 7-10 minutes on alternating days for two consecutive weeks on an inclined treadmill with an incline of 14 degrees and a pace of 1.7 miles per hour. At the end of each week, the subject provided a urine sample for the comparison of CTX-II levels to baseline. An obese male (age 43) with BMI >25 kg/m² and having healthy knee joints (no resting knee pain or stiffness) who exercised fewer than 2 times per week provided a urine sample for basal CTX-II level determination. The subject subsequently performed exercise of 50 stairs (standard height) per leg over approximately 10 minutes daily for one week.

Collectively, this data indicated that it was indeed possible to induce meaningful increases in uCTX-II in articularly healthy individuals *via* a variety of exercises, and that even moderate-intensity exercise resulted in measurable increases in uCTX-II in suspected cartilage-sensitive individuals (i.e. post-menopausal and/or obese). With this as a basis, we set out to more formally and systematically evaluate the effects of different moderate-intensity exercises on uCTX-II in healthy, post-menopausal women.

Non-interventional clinical phase evaluating design variables

A non-interventional clinical trial was conducted utilizing the services of a clinical contract research organization (QPS Bio-Kinetic; Springfield, MO USA) and the exercise facilities of a nearby hospital (The Meyer Orthopedic & Rehabilitation Center-Cox Health Systems; Springfield, MO USA). The study was conducted in accordance with the U.S. Food & Drug Administration's principles of Good Clinical Practice (Title 21, Code of Federal Regulations, Parts 50 & 56 and ICH E6) and the Declaration of Helsinki (1996 version). The study protocol was approved by a duly authorized Institutional Review Board (IRB) and all subjects provided their written informed consent in order to participate.

A group of 30 post-menopausal females (age range 46-72) with healthy knee joints (no resting knee pain or stiffness), all of whom exercised fewer than 2 times per week, were randomized into one of three different low-impact, moderate-intensity exercise regimen groups. Group A subjects walked for a minimum of 7 minutes on

alternating days for two consecutive weeks on a 14 degree inclined treadmill at an approximate pace of 1.7 miles per hour. Group B subjects performed exercise for a minimum of 7 minutes on alternating days for two consecutive weeks on a seated step machine (NuStep® brand) with a workload of 7.0 and a pace of 30-40 steps per minute. Group C subjects performed 3 sets of 8 lifts each of 90 pounds (41 kg) on a seated leg press (Cybex® brand) in a maximum of 7 minutes on alternating days for two consecutive weeks. All subjects provided urine samples for basal CTX-II level determination and urine samples at the end of each week for comparison of CTX-II levels to baseline. Urine samples were obtained from the 2nd void of the morning collected within 12-24 hours after each subject completed the final exercise for the week. Samples were frozen (-20°C) immediately following collection until needed for assaying.

Adverse events

The participants' self-assessment diaries were reviewed, and the subjects were interviewed at each clinic visit so that any discomfort beyond what would normally be expected for moderate exercise or other adverse events were recorded and reported in accordance with applicable FDA regulations.

With many of the model design questions answered, it was then time to assess whether the model was sensitive enough to enable the evaluation of joint therapeutics. That is, would the change in uCTX-II induced by exercise be sufficiently large to be able to realize a chondroprotective effect resulting from a joint therapeutic.

Pilot Interventional clinical phase testing the trial design

Following a three week resting period, the same groups of 30 post-menopausal females were re-randomized into one of two different treatment groups. In this phase of the study, all subjects performed the same low-impact, moderate-intensity exercise regimen (3 sets of 8 lifts each of 90 pounds (41 kg) on a seated leg press (Cybex® brand) in a maximum of 7 minutes on alternating days for two consecutive weeks). Group 1 subjects consumed one 500 mg capsule per day of a powdered eggshell membrane joint therapeutic composition (commercially available as NEM® brand eggshell membrane; ESM Technologies, LLC, Carthage, MO USA) for 7 days prior to beginning the exercise regimen and continued to take the treatment during the two-week exercise period (3 weeks total). Group 2 subjects consumed one 500 mg capsule per day of the same powdered eggshell membrane joint therapeutic composition but began taking it on day 1 of the two-week exercise period and continued to do so throughout the remaining time (2 weeks total). As in period 1 of the study, all subjects provided urine samples for basal CTX-II level determination and urine samples at the end of each week for the comparison of CTX-II levels to baseline.

Adverse events

The participants' self-assessment diaries were reviewed, and the subjects were interviewed at each clinic visit so that any discomfort beyond what would normally be expected for moderate exercise or other adverse events were recorded and reported in accordance with applicable FDA regulations.

Results

Initial investigation of design variables

The results of the temporal clearance evaluation for the two females (ages 34 & 37) and one male (age 34) having healthy knee joints (no resting knee pain or stiffness) and one female (age 60) with diagnosed OA of the right knee all with a BMI < 25 kg/m² are reported in Figure 1. There appears to be a reproducible initial increase in uCTX-II within the first 2-4 hours following exercise, followed by somewhat of a plateau. However, for all subjects, the maximum (or near maximum) level of uCTX-II was observed 24 hours post-exercise (2nd void of the morning). Not surprisingly, the female with OA had the highest basal uCTX-II and appeared to be the most sensitive to joint strain resulting from exercise.

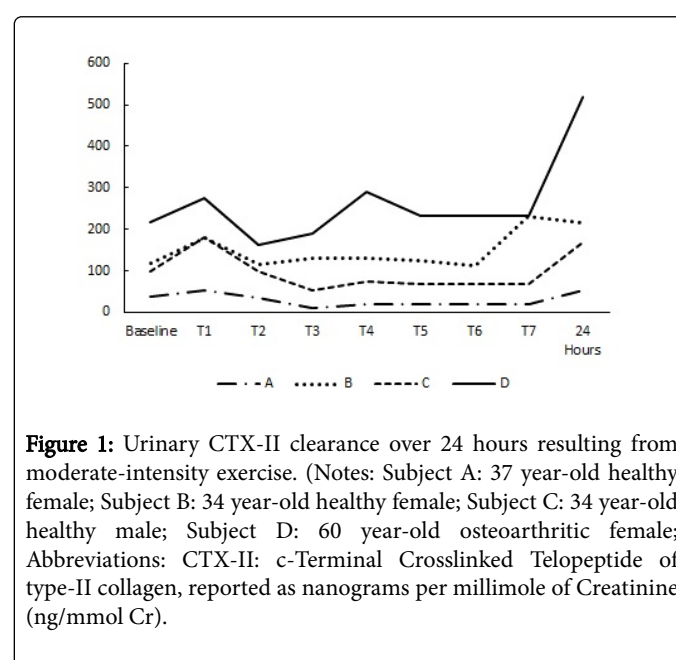


Figure 1: Urinary CTX-II clearance over 24 hours resulting from moderate-intensity exercise. (Notes: Subject A: 37 year-old healthy female; Subject B: 34 year-old healthy female; Subject C: 34 year-old healthy male; Subject D: 60 year-old osteoarthritic female; Abbreviations: CTX-II: c-Terminal Crosslinked Telopeptide of type-II collagen, reported as nanograms per millimole of Creatinine (ng/mmol Cr).

For the post-menopausal female (age 67) with healthy knee joints who did not exercise regularly, CTX-II had increased from baseline (156 ng/mmol Cr) by 20.4% at week 1 (188 ng/mmol Cr) and at week 2 CTX-II (199 ng/mmol Cr) was slightly further elevated at 27.1% above baseline. For the obese male (age 43) with BMI > 25 kg/m² and having healthy knee joints (no resting knee pain or stiffness) who exercised fewer than 2 times per week basal CTX-II was 70 ng/mmol Cr. On Day 4, CTX-II (100 ng/mmol Cr) had increased by 43.6% from baseline and on day 8, CTX-II (87 ng/mmol Cr) was increased by a lesser 24.3% from baseline.

Non-interventional clinical phase evaluating design variables

Table 1 contains baseline demographic data for the three groups (A, B & C) of post-menopausal females from the 30-subject non-interventional clinical phase. The three groups were not statistically different in any of the baseline demographic data using the non-parametric Kruskal-Wallis Test for multiple groups. It is important to note that their urinary CTX-II levels are consistent with being healthy post-menopausal females, having average uCTX-II levels at the very lowest end of the range of what would be expected for arthritic

subjects [11,12] and well within the expected normal range for women of their age, BMI and hormonal status [10].

	Group A	Group B	Group C
Age (yrs)	58.2 ± 4.7	52.4 ± 6.2	57.8 ± 7.1
Weight (kg)	73.4 ± 11.1	83.5 ± 18.2	77.2 ± 12.2
BMI	26.8 ± 3.4	30.1 ± 4.4	28.6 ± 4.2
uCTX-II (ng/mmol Cr)	259 ± 102	219 ± 98	288 ± 84
Notes: Values are reported as mean ± standard deviation (n=10 per group). There were no statistical differences between treatment groups in any of the listed parameters. Abbreviations: BMI: Body Mass Index; calculated as weight in kilograms divided by the square of height in meters. uCTX-II: urinary c-Terminal Crosslinked Telopeptide of type-II collagen, reported as nanograms per millimole of Creatinine (ng/mmol Cr).			

Table 1: Period 1 participant baseline demographic data.

Table 2 contains urinary CTX-II results for the test groups of subjects (Group A not shown) at baseline and after performing the designated exercise regimens for two consecutive weeks.

	Time Point	uCTX-II (% change)	Immediate Pain (% change)	12-hour Pain (% change)	Immediate Stiffness (% change)	12-hour Stiffness (% change)
Group B	Baseline	219 ± 98 (N/A)	0.1 ± 0.3 (N/A)	0.1 ± 0.3 (N/A)	0.2 ± 0.4 (N/A)	0.2 ± 0.4 (N/A)
	Week 1	233 ± 103 (+6.4) [#]	0.6 ± 1.0 (+500) [#]	0.7 ± 1.1 (+600) [*]	0.9 ± 1.0 (+350) [#]	0.9 ± 1.0 (+350) [#]
	Week 2	218 ± 102 (-0.5) [#]	0.8 ± 1.5 (+700) [#]	0.8 ± 1.1 (+700) [*]	0.6 ± 1.1 (+200)	0.9 ± 1.1 (+350) [#]
Group C	Baseline	288 ± 84 (N/A)	0.1 ± 0.3 (N/A)	0.1 ± 0.3 (N/A)	0.3 ± 0.5 (N/A)	0.3 ± 0.5 (N/A)
	Week 1	331 ± 117 (+14.9) [*]	1.0 ± 0.8 (+900) [*]	1.0 ± 1.1 (+900) [*]	1.0 ± 0.8 (+233) [*]	1.2 ± 1.0 (+300) [*]
	Week 2	313 ± 108 (+8.7) [*]	0.8 ± 0.8 (+700) [*]	0.7 ± 1.1 (+600) [#]	1.2 ± 1.0 (+300) [*]	0.9 ± 1.1 (+233) [#]
Notes: Values are reported as mean ± standard deviation (% Change from baseline) (n=10 per group). Group B performed seated step machine exercise. Group C performed seated leg press exercise. Abbreviations: uCTX-II: urinary c-Terminal Crosslinked Telopeptide of type-II collagen, reported as nanograms per millimole of Creatinine (ng/mmol Cr). [*] p<0.05; [#] p~0.10 versus baseline.						

Table 2: Pain, Stiffness, and uCTX-II levels at baseline and after 1 week & 2 weeks of performing one of two low-impact, moderate-intensity exercise regimens.

Using the non-parametric Friedman Test for repeated measures coupled with a post-hoc Conover analysis to determine which time points (Baseline, Week 1, or Week 2) differed, it was determined that Group C differed statistically (p<0.05) at both week 1 (331 ± 117) and week 2 (313 ± 108) from baseline (288 ± 84). Group B showed a clear statistical trend (p<0.10) for week 1 & week 2 to differ from Baseline. Group A was not statistically different at any time point.

Only one or two subjects in Group A (not shown) experienced any pain or stiffness from walking on an inclined treadmill (as described previously). Pain and stiffness results for Group B and Group C are also reported in Table 2. There were substantial increases in pain in both groups both immediately after exercise (500%-900%) and 12 hours post-exercise (600%-900%). Similarly, there were substantial increases in stiffness in both groups both immediately after exercise (200%-350%) and 12 hours post-exercise (233%-350%). For Group B, these results were statistically significant (p<0.05) for 12-hour post-exercise pain (week 1 & week 2) and showed a statistical trend (p~0.10) for immediate pain (both weeks), immediate stiffness (week

1), and 12-hour post-exercise stiffness (both weeks). For Group C, these results were statistically significant (p<0.05) for all categories (immediate pain & stiffness and 12-hour post-exercise pain & stiffness) at Week 1 and for week 2 immediate pain & stiffness and showed a statistical trend (p~0.10) for 12-hour post-exercise pain & stiffness.

Adverse events

No serious adverse events were reported in this study period. There were twelve Adverse Events (AEs) reported in period 1 including: (7) headaches and one instance each of congestion, cold-like symptoms, sore throat, nausea, and leg cramp. No AEs required discontinuation of the exercise regimen nor did they lead to withdrawal from the study.

Pilot interventional clinical phase testing the trial design

Table 3 contains the baseline demographic data for the two groups of re-randomized study subjects from the interventional clinical phase

(Period 2). The groups were not statistically different in any of the baseline demographic data using the non-parametric Mann-Whitney U Test for independent groups. It is also significant that their uCTX-II levels had returned to levels similar to what was found in Period 1 of the study following the 3-week resting period. Again, CTX-II levels

remained consistent with being healthy post-menopausal females. Table 4 presents the uCTX-II results for the two groups of subjects at baseline and after performing the designated exercise regimen for two consecutive weeks.

	Group 1	Group 2
Age (yrs)	56.2 ± 8.0	56.1 ± 4.8
Weight (kg)	78.4 ± 12.1	77.7 ± 16.7
BMI	28.4 ± 3.7	28.6 ± 4.7
uCTX-II (ng/mmol Cr)	220 ± 97	174 ± 76
Notes: Values are reported as mean ± standard deviation (n=15 per group). There were no statistical differences between treatment groups in any of the listed parameters. Abbreviations: BMI: Body Mass Index, calculated as weight in kilograms divided by the square of height in meters. uCTX-II: urinary c-Terminal Crosslinked Telopeptide of type-II collagen, reported as nanograms per millimole of Creatinine (ng/mmol Cr).		

Table 3: Period 2 participant baseline demographic data.

There were no statistical differences for either group versus baseline for urinary CTX-II, which is in contrast to results found in Group C in Study Period 1 (performing the same exercise regimen) (Table 2), indicating that the eggshell membrane joint therapeutic composition is chondroprotective (cartilage-sparing) in this clinical trial designed to induce cartilage turnover *via* exercise.

Because there were also no statistically significant differences between Group 1 and Group 2 for any of the pain or stiffness categories (Table 4), the same 10 subjects from Group C were compared directly to corresponding results obtained in Study Period 1 performing the same exercise regimen when not consuming a joint therapeutic composition (Table 5). There were large reductions in pain

and stiffness absolute treatment effects (-258% to -867%) from consuming the NEM joint therapeutic while performing the exercise regimen in study period 2 versus performing the same exercise regimen while untreated in study period 1. These differences were statistically significant (p<0.05) for Week 1 12-hour pain and for week 2 both Immediate & 12-hour stiffness. All other pain and stiffness criteria failed to reach statistical significance, however they all showed trends for improvement (p<0.10). There were moderate reductions (-11.7% to -19.1%) in the uCTX-II absolute treatment effect as well, however this failed to reach statistical significance. Week 2 uCTX-II did, however, show a trend for improvement (p<0.10).

	Time Point	uCTX-II (% change)	Immediate Pain (% change)	12-hour Pain (% change)	Immediate Stiffness (% change)	12-hour Stiffness (% change)
Group 1	Baseline	220 ± 97 (N/A)	0.4 ± 1.1 (N/A)	0.4 ± 1.1 (N/A)	0.5 ± 1.0 (N/A)	0.5 ± 1.0 (N/A)
	Week 1	222 ± 92 (+1.3)	1.2 ± 1.9 (+200)	1.1 ± 1.7 (+168)	0.8 ± 1.3 (+50)	1.0 ± 1.7 (+88)
	Week 2	209 ± 93 (-4.8)	0.7 ± 1.4 (+83)	0.6 ± 1.0 (+50)	0.7 ± 1.2 (+25)	0.7 ± 1.0 (+25)
Group 2	Baseline	174 ± 76 (N/A)	0.4 ± 0.7 (N/A)	0.4 ± 0.7 (N/A)	0.6 ± 1.0 (N/A)	0.6 ± 1.0 (N/A)
	Week 1	178 ± 87 (+2.3)	0.9 ± 0.8 (+117)	0.7 ± 0.9 (+83)	0.9 ± 0.9 (+44)	0.8 ± 1.1 (+33)
	Week 2	161 ± 58 (-5.7)	0.9 ± 0.7 (+117)	0.3 ± 0.6 (-17)	0.6 ± 0.6 (0)	0.5 ± 0.7 (-11)
Notes: Values are reported as mean ± standard deviation (% change from baseline) (n= 15 per group). Abbreviations: uCTX-II: urinary c-Terminal Crosslinked Telopeptide of type-II collagen, reported as nanograms per millimole of Creatinine (ng/mmol Cr). *p<0.05; # p~0.10 versus baseline.						

Table 4: Pain, stiffness and uCTX-II levels at baseline and after 1 week & 2 weeks of performing a low-impact, moderate-intensity exercise regimen while consuming a joint therapeutic composition.

Adverse events

No serious adverse events were reported in this study period. There were twenty-three AEs reported in period 2 including: (13) headaches, (4) cold-like symptoms, and one instance each of constipation, sore

throat, nausea, shoulder pain, swelling, and leg cramp. No AEs required discontinuation of the treatment product nor did they lead to withdrawal from the study.

	Weeks	Treatment		Absolute
	Post-treatment	Untreated	NEM	Treatment Effect
uCTX-II	Baseline (n=10, 10)	288 ± 84	237 ± 82	-
	1 (n=10, 10)	331 ± 117	227 ± 85	-19.10%
	2 (n=10, 10)	313 ± 108	230 ± 70	-11.7%#
Immediate	Baseline (n=10, 10)	0.1 ± 0.3	0.6 ± 1.0	-
Pain	1 (n=10, 10)	1.0 ± 0.8	0.8 ± 0.9	-867%#
	2 (n=10, 10)	0.8 ± 0.8	0.7 ± 0.8	-683%#
12-hour	Baseline (n=10, 10)	0.1 ± 0.3	0.6 ± 1.0	-
Pain	1 (n=10, 10)	1.0 ± 1.1	0.7 ± 0.9	-883%*
	2 (n=10, 10)	0.7 ± 1.1	0.4 ± 0.7	-633%#
Immediate	Baseline (n=10, 10)	0.3 ± 0.5	0.8 ± 1.1	-
Stiffness	1 (n=10, 10)	1.0 ± 0.8	0.9 ± 1.1	-220%#
	2 (n=10, 10)	1.2 ± 1.0	0.6 ± 0.8	-325%*
12-hour	Baseline (n=10, 10)	0.3 ± 0.5	0.8 ± 1.1	-
Stiffness	1 (n=10, 10)	1.2 ± 1.0	0.9 ± 1.3	-287%#
	2 (n=10, 10)	0.9 ± 1.1	0.6 ± 0.8	-258%*

Notes: Values are reported as mean ± standard deviation. Absolute Treatment Effect is the net difference of NEM treatment versus untreated for the change in mean treatment effect from baseline expressed as a percent. Negative values for pain or function indicate superior improvement in the treatment group.

Abbreviations: *p<0.05; #p<0.10 versus untreated

Table 5: Urinary CTX-II, pain, and stiffness levels at baseline and after 1 week & 2 weeks of performing a low-impact, moderate-intensity exercise regimen while untreated and while consuming a joint therapeutic composition.

Discussion

Articular joint diseases are very prevalent throughout the world, particularly in adults 60 years of age and older, and result in significant costs (both financial and quality-of-life) for those that suffer from the debilitating diseases. Owing to the lack of drugs that can halt the progression of arthritis (be disease modifying) there is an obvious current need for additional joint therapeutics. We report here the development of a clinical trial design to aid in the evaluation of chondroprotective joint therapeutics.

Initial design aspects were investigated, and it was found that exercise-induced urinary CTX-II is excreted similarly over time in various healthy and arthritic individuals (i.e. young adult females, post-menopausal non-OA and OA females, young adult normal weight and obese males). The levels of uCTX-II varied somewhat over time following exercise, however collecting the 2nd void of the morning approximately 24 hours post-exercise possibly smooths some of the individual variation in protein clearance and appears to reproducibly generate maximal uCTX-II values. Interestingly, uCTX-II levels return to baseline within 48-72 hours following exercise. Therefore, it is important that the subjects exercise repeatedly (e.g. every day, alternating days, etc.) to increase the likelihood of capturing the chondroprotective effect from a joint therapeutic under investigation.

The cartilage of various healthy subsets of the population (no joint disease or symptoms) was found to be sensitive to low-impact, low-

intensity exercise *via* the timely production and urinary excretion of CTX-II. This differs markedly from previous reports [9] of elevated uCTX-II levels resulting from high-impact, high-intensity exercise (i.e. cross-country runners) but not in low-impact, high-intensity exercises (i.e. crew rowers or competitive swimmers). We believe that this difference may be attributable to an inherent sensitivity to strain in the cartilage of either post-menopausal women or infrequent exercisers, or a combination of these factors. This difference may also be attributable to the joints of the subjects adjusting and adapting to the exercise strain after sufficient repetition, as would occur in endurance athletes. In the present design, we hoped to exploit both factors and maximize the likelihood of achieving measurable exercise-induced cartilage effects by utilizing infrequently-exercising, post-menopausal women as study subjects.

Cartilage is primarily composed of Extracellular Matrix (ECM), a composite network of proteins such as type-II collagen interacting with negatively charged polysaccharides such as hyaluronic acid and chondroitin sulfate (usually present as proteoglycans), all of which are synthesized and secreted by chondrocytes. During normal cartilage turnover in healthy articular joints ECM production balances ECM breakdown thereby ensuring the continuous renewal of this critical joint-cushioning tissue. However, pathological conditions such as arthritis are characterized by an imbalance in cartilage turnover, in which catabolic processes predominate over anabolic processes. ECM synthesis cannot keep pace with degradation and a loss of the

structural integrity of the articular cartilage results. Products of this degradation imbalance can be found in both blood and urine of arthritic patients. Initially cartilage breakdown occurs *via* slow but extensive proteoglycan loss in the ECM matrix resulting in loss of cartilage thickness. The process culminates in the breakdown of the fibrillar collagen support matrix and ultimately leads to chondrocyte apoptosis. Proteoglycan loss has been shown to be reversible in a number of canine studies involving induced OA, however collagen matrix degradation appears to be irreversible [13]. Therefore, it is critical for a chondroprotective joint therapeutic to spare the collagen matrix within cartilage, and this effect can be demonstrated *via* monitoring of uCTX-II a collagen fragment generated directly from the degradation of the cartilage collagen matrix.

We first set out to evaluate different types of exercise and possible effects these might have on cartilage turnover in these subjects. The exercises were chosen to provide some strain on the knee. That is, walking on an inclined treadmill was chosen as opposed to walking on a flat surface to provide at least some level of additional knee strain. However, the exercises were carefully selected specifically to avoid significant knee joint impact. This was done not only to isolate the effect of joint strain versus joint impact on cartilage, but also to make the exercises more amenable to performance by older study subjects. When reviewing data from the non-interventional period of the study across all groups, there is a clear trend for the strenuous nature of the exercise (lifting weight>seated step machine>inclined treadmill) to affect the magnitude of the cartilage turnover of the study subjects. In fact, walking on an inclined treadmill appeared to be beneficial in reducing cartilage turnover. This unexpected finding resulting from joint strain compared to the known effect from the nature of exercise impact (low-versus high) on the joint not only enables the evaluation of exercise induced cartilage turnover from low-intensity exercise in an older target population, but may also inform future designs of exercise for joint rehabilitation. If indeed the loss of the cartilage collagen matrix is irreversible in human joints as has been shown in a canine model [13], care should be taken to limit both joint impact and strain while rehabilitating a joint following injury and/or surgery. Further research will be needed to determine if this concern is borne out.

We report here the first evidence to suggest that this clinical trial design can be used to evaluate the chondroprotective efficacy of joint therapeutics. Subjects were given the joint therapeutic (natural eggshell membrane; NEM) either 1 week prior to or commencing with the exercise regimen. No differences were evident in this scenario. Both groups had a small (<2%) increase in uCTX-II in the first week with a subsequent moderate (<6%) decrease noted in the second week. These results are in contrast to the increases realized with subjects performing the same exercise regimen while untreated (week 1+14.9%, week 2+8.7%). When comparing the same ten subjects from both periods of the study (treated versus untreated), the net treatment effect on uCTX-II was -19.1% for week 1 and -11.7% for week two. Future research will require a substantially greater number of study subjects to ensure the ability to detect statistically significant differences at this treatment effect size. Despite the fact the design was intended to facilitate the evaluation of chondroprotection, it appears to potentially be sufficiently responsive to simultaneously evaluate joint therapeutic effects on symptomatology (i.e. pain and stiffness), as well. Again, comparing the ten subjects that performed the same exercise regimen both treated and untreated showed a number of statistically significant treatment effects for both joint pain and stiffness (i.e. 12-hour pain and both immediate and 12-hour stiffness). This topic warrants further validation of the design.

As the study was intended to investigate design variables, it suffered from a number of limitations. The limited enrollment (30 subjects) hindered the determination of statistical significance and may have limited realization of further clinically meaningful results. The inclusion of a comparative treatment agent would likely have provided additional information but would have required a significantly larger initial study population. A follow-up randomized, double-blind, placebo-controlled study with some minor modifications will allow us to better validate the current study design and may provide additional valuable insight into the use of uCTX-II in evaluating chondroprotective joint therapeutics in healthy individuals. Importantly, based on results from our preliminary investigation this design would be expected to function equally well, if not better, in an OA population wherein their cartilage would be even more sensitive to the effects of exercise [14].

Conclusion

With one third of those 65 and older in the U.S. having been diagnosed with osteoarthritis, and that number expected to grow immensely as the overall population ages, it is important for patients to have treatment options that are both effective and safe. With the current lack of effective disease modifying drugs (i.e. DMOADs) and with existing drugs having significant safety issues (i.e. DMARDs), there is a substantial need to improve our ability to develop joint therapeutics to meet current and future demands. The clinical trial design presented here shows great potential to evaluate chondroprotective joint therapeutics in healthy individuals, where sparing cartilage may very well prevent patients from ultimately developing arthritis. The design also demonstrated the potential to evaluate joint therapeutic efficacy in improving symptomatology (i.e. joint pain and stiffness). By extension, this design may also enable the evaluation of chondroprotective joint therapeutics in an OA population, particularly where cartilage preservation has reached a critical stage.

Acknowledgements

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Disclosure

The study sponsor was ESM Technologies, LLC. KJR & MB are employed by the sponsor.

DM & SAD are employed by the CRO and have no other competing interests.

References

1. Woolf AD, Pfleger B (2003) Burden of Major Musculoskeletal Conditions. Bull World Health Organ 81: 646-656.
2. Wieland HA, Michaelis M, Kirschbaum BJ, Rudolphi KA (2005) Osteoarthritis - An Untreatable Disease? Nat Rev Drug Discov 4: 331-334.
3. Ruderman EM (2012) Overview of safety of non-biologic and biologic DMARDs. Rheumatol 51: vi37-vi43.
4. Ishii H, Tanaka H, Katoh K, Nakamura H, Nagashima M, et al. (2002) Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis. Osteoarthritis Cartilage 10: 277-281.

5. Mishra R, Singh A, Chandra V, Negi MPS, Tripathy BC, et al. (2012) A comparative analysis of serological parameters and oxidative stress in osteoarthritis and rheumatoid arthritis. *Rheumatol Int* 32: 2377-2382.
6. Punzi L, Calò L, Plebani M (2002) Clinical Significance of Cytokine Determination in Synovial Fluid. *Crit Rev Clin Lab Sci* 39: 63-88.
7. Belcher C, Yaqub R, Fawthrop F, Bayliss M, Doherty M (1997) Synovial fluid chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in arthritic and normal knees. *Ann Rheum Dis* 56: 299-307.
8. Bauer DC, Hunter DJ, Abramson SB, Attur M, Corr M, et al. (2006) Classification of osteoarthritis biomarkers: a proposed approach. *Osteoarthritis Cartilage* 14: 723-727.
9. O’Kane JW, Hutchinson E, Atley LM, Eyre DR (2006) Sport-related differences in biomarkers of bone resorption and cartilage degradation in endurance athletes. *Osteoarthritis Cartilage* 14: 71-76.
10. Mouritzen U, Christgau S, Lehmann HJ, Tankó LB, Christiansen C (2003) Cartilage turnover assessed with a newly developed assay measuring collagen type II degradation products: influence of age, sex, menopause, hormone replacement therapy, and body mass index. *Ann Rheum Dis* 62: 332-336.
11. Garnero P, Piperno M, Gineyts E, Christgau S, Delmas PD, et al. (2001) Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. *Ann Rheum Dis* 60: 619-626.
12. Karsdal MA, Byrjalsen, Bay-Jensen AC, Henriksen K, Riis BJ, et al. (2010) Biochemical markers identify influences on bone and cartilage degradation in osteoarthritis - the effect of sex, Kellgren-Lawrence (KL) score, Body Mass Index (BMI), oral salmon calcitonin (sCT) treatment and diurnal variation. *BMC Musculoskel Disord* 11: 125.
13. Brandt KD (2003) Response of joint structures to inactivity and to reloading after immobilization. *Arthritis Rheum* 49: 267-271.
14. Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, et al. (2008) Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. *Arthritis Rheum* 58: 26-35.

Beneficial effects of natural eggshell membrane versus placebo in exercise-induced joint pain, stiffness, and cartilage turnover in healthy, postmenopausal women

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Purpose: Despite its many health benefits, moderate exercise can induce joint discomfort when done infrequently or too intensely even in individuals with healthy joints. This study was designed to evaluate whether NEM[®] (natural eggshell membrane) would reduce exercise-induced cartilage turnover or alleviate joint pain or stiffness, either directly following exercise or 12 hours post exercise, versus placebo.

Patients and methods: Sixty healthy, postmenopausal women were randomly assigned to receive either oral NEM 500 mg (n=30) or placebo (n=30) once daily for two consecutive weeks while performing an exercise regimen (50–100 steps per leg) on alternating days. The primary endpoint was any statistically significant reduction in exercise-induced cartilage turnover via the biomarker C-terminal cross-linked telopeptide of type-II collagen (CTX-II) versus placebo, evaluated at 1 and 2 weeks of treatment. Secondary endpoints were any reductions in either exercise-induced joint pain or stiffness versus placebo, evaluated daily via participant questionnaire. The clinical assessment was performed on the per protocol population.

Results: NEM produced a significant absolute treatment effect (TE_{abs}) versus placebo for CTX-II after both 1 week (TE_{abs} -17.2%, $P=0.002$) and 2 weeks of exercise (TE_{abs} -9.9%, $P=0.042$). Immediate pain was not significantly different; however, rapid treatment responses were observed for immediate stiffness (Day 7) and recovery pain (Day 8) and recovery stiffness (Day 4). No serious adverse events occurred and the treatment was reported to be well tolerated by study participants.

Conclusion: NEM rapidly improved recovery from exercise-induced joint pain (Day 8) and stiffness (Day 4) and reduced discomfort immediately following exercise (stiffness, Day 7). Moreover, a substantial chondroprotective effect was demonstrated via a decrease in the cartilage degradation biomarker CTX-II. Clinical Trial Registration number: NCT02751944.

Keywords: chondroprotective, CTX-II, cartilage degradation, breakdown

Introduction

Exercise is an integral component of a healthy lifestyle and is regularly practiced by hundreds of millions of people throughout the world. It is recommended that adults should complete 30 minutes or more of moderate-intensity (aerobic) physical activity every – or nearly every – day.¹ Exercise is known to impart a multitude of health benefits, particularly in the prevention of a number of chronic diseases such as cardiovascular disease² and type II diabetes³ and reduces the incidence and detrimental effects of obesity,⁴ which is known to contribute to the development of these

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and other chronic diseases. As people age, physical activity gains even greater importance with the need to maintain articular joint flexibility, lessen frailty, and improve balance to reduce the risk of falls⁵ that can lead to increased disability and mortality rates in older adults.^{6,7} Numerous studies have demonstrated the beneficial effects of exercise in relieving osteoarthritis (OA) symptoms,⁸ particularly for weight-bearing joints such as the knees or hips, and exercise is recommended by the American College of Rheumatology as a nonpharmacologic therapy for OA.⁹ There is even some clinical evidence to suggest that exercise may play a role in the prevention of OA.¹⁰

Despite the fact that articular joints are designed for and intended to move, moderate exercise can still induce discomfort in joints when done infrequently, or too intensely, or with too great a load, or for too long a period. This discomfort is often realized as either pain or stiffness in the joint that was the focus of the exercise. Whether this short-term insult to the joint and connective tissues leads to degenerative disease over the long-term remains to be answered definitively. What is clear is that lack of physical activity (immobility) has significant detrimental effects (morphological, biochemical, and biomechanical) on all joint tissues (eg, ligaments, tendons, cartilage, synovial fluid, joint capsule, and bone). Ligament fibers lose integrity and weaken biomechanically, periarticular muscles and associated tendons atrophy destabilizing the joint, articular cartilage atrophies (loss of cartilage thickness) and lesions form at points of contact, hyaluronic acid content of synovial fluid lessens reducing its lubricating ability, fibro-fatty connective tissue proliferates from the joint capsule (synovium) into the joint space creating adhesions, and periarticular bones become osteoporotic and fragile.¹¹ Fortunately, all of these negative effects are reversible given sufficient time and gradual reloading during the resumption of physical activity.¹² Interestingly, many of the detrimental effects seen with joint immobility or lack of physical activity mirror the morphological, biochemical, and biomechanical changes seen in the pathology of OA.¹²

Cartilage is primarily composed of extracellular matrix (ECM), a composite network of proteins such as type-II collagen interacting with negatively charged polysaccharides such as hyaluronic acid and chondroitin sulfate, all of which are synthesized and secreted by chondrocytes. During normal cartilage turnover (metabolism) in healthy articular joints, ECM production balances ECM breakdown, thereby ensuring the continuous renewal of this critical joint-cushioning tissue. However, pathologic conditions such as OA are characterized by an imbalance in cartilage turnover, in which catabolic

processes predominate over anabolic processes. ECM synthesis cannot keep pace with degradation and a loss of structural integrity of articular cartilage results. This cartilage metabolism imbalance coupled with biomechanical stress in the joint leads to chronic inflammation and ultimately irreversible joint destruction. These cartilage degradation products can be found in the blood and urine of both healthy and arthritic subjects.

A number of biomarkers of cartilage turnover have been investigated for their diagnostic and prognostic properties.¹³ Of these biomarkers, C-terminal cross-linked telopeptide of type-II collagen (CTX-II), a marker of cartilage degradation, has shown the most potential. It has been associated with both the incidence and progression of OA in multiple clinical trials^{14,15} and is predictive of the progression of OA both radiographically,¹⁶ including two ≥ 5 year longitudinal studies,^{17,18} and by MRI.¹⁹ Urinary CTX-II (uCTX-II) levels are known to be substantially elevated in those afflicted with articular joint diseases like OA and rheumatoid arthritis (RA), but levels are also known to be elevated in a variety of healthy subsets of the population, as well. For example, uCTX-II levels in growing children are about 50-fold higher than that of adults.²⁰ uCTX-II levels have been shown to be elevated because of high-impact, strenuous exercise in healthy college-aged endurance athletes such as cross-country runners by about 85% over age- and weight-matched controls, but were not significantly elevated in lower-impact endurance athletes like swimmers and rowers.²¹ uCTX-II has also been shown to be about twofold higher in postmenopausal women versus age-matched premenopausal women and moderately elevated (~25%) in overweight persons (body mass index [BMI], ≥ 25 kg/m²) versus normal weight controls (BMI < 25 kg/m²).²²

Eggshell membrane (ESM), found between the calcified shell and the albumin in chicken eggs, is primarily composed of fibrous proteins such as collagen Type I,²³ which form the mesh-like structure of the bilayered material. ESMs have also been shown to contain other bioactive components, namely, glycosaminoglycans (ie, dermatan sulfate, chondroitin sulfate, hyaluronic acid, etc).²⁴ ESM is known to reduce the expression of various pro-inflammatory cytokines, including the key mediators of inflammation interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) both in vitro²⁵ and in vivo.²⁶ A proprietary form of ESM, commercially available as the branded product NEM[®] (natural eggshell membrane); ESM Technologies, LLC, Carthage, MO, USA), has demonstrated safety and efficacy in multiple clinical trials in relieving joint pain and stiffness in humans

with OA^{27–29} and has been shown to reduce CTX-II levels in rat models of both OA³⁰ and RA³¹ and in naturally occurring joint disease in dogs.³²

Because of the importance of exercise to overall health and disease prevention particularly as it relates to the long-term health of joints and joint disease, there is a need to evaluate therapeutics that can not only protect joints from the potential detrimental effects from exercise but also reduce the resultant pain and stiffness from exercise thereby encouraging adherence to a regular exercise regimen. Taking into account the apparent sensitivity of articular cartilage to increased joint strain in postmenopausal women, a 2-week randomized controlled trial was conducted to evaluate the effects of NEM brand ESM on exercise-induced joint pain, stiffness, and cartilage turnover in healthy, postmenopausal women. The results are presented herein.

Patients and methods

Study design

This study was conducted utilizing the services of a clinical contract research organization (QPS Bio-Kinetic, Springfield, MO, USA) according to a single-center, randomized, double-blind, placebo-controlled, parallel-group design in accordance with the US Food and Drug Administration (FDA)'s principles of Good Clinical Practice (Title 21, Code of Federal Regulations, Parts 50 and 56 and ICH E6) and the Declaration of Helsinki (1996 version) (Clinical Trial Registration Number NCT02751944). The study protocol was approved by a registered institutional review board (IRB) (Bio-Kinetic Clinical Applications IRB #1; Reg No IRB00002771) and subjects provided their written informed consent in order to participate. After meeting all inclusion/exclusion criteria at screening, eligible subjects were then randomized (1:1) to receive either NEM or placebo in the order in which they were enrolled in the study using a permuted block randomization table consisting of four subjects per block. Treatment consisted once-daily orally of either 500 mg of NEM or placebo. Treatment compliance was checked at clinic visits by participant interview and by counting the number of unused doses of the study capsules. Acetaminophen was allowed for pain relief rescue, if necessary. Subjects recorded the time and amount of acetaminophen taken in subject diaries. All participants, clinical staff, and study management staff remained blinded to treatment assignment throughout the study.

The intent of this study was to evaluate whether NEM brand ESM would reduce cartilage turnover (via the CTX-II biomarker) or alleviate joint pain or stiffness, either directly

following exercise or 12 hours post exercise (recovery), versus placebo. Participants performed 50–100 steps per leg utilizing a 6"-tall aerobics step at the clinical site. At screening, subjects performed 50 steps per leg, increasing by 10 at a time per leg up to 100 steps per leg, until they experienced at least a 1 unit change in discomfort (either pain or stiffness) from their resting rating. This number of steps was then assigned to that subject for the remainder of the study. Participants then followed their assigned exercise regimen on alternating days for 2 consecutive weeks (ie, Sunday, Tuesday, Thursday, Saturday, Monday, Wednesday, and Friday). Additionally, participants were required to provide blood and urine samples approximately 24 hours after the final weekly exercise visit, that is, on Friday of Week 1 and Saturday of Week 2. Changes in pain and stiffness (both immediate and 12-hour) and changes in CTX-II levels were compared to those of the placebo group.

Patients

All healthy postmenopausal women, 40–75 years of age, who had not been diagnosed with a joint or connective tissue disorder of the lower extremities (ie, ankles, knees, or hips), were considered for enrollment in the study. To be eligible to participate, subjects must have been amenorrheic (postmenopausal) for at least 12 months prior to baseline evaluation, either naturally or surgically. Additionally, subjects must not have had persistent lower-extremity joint pain at rest; however, subjects could have experienced mild periodic lower-extremity joint pain rated ≤ 3 on a 10-point ordinal scale. Subjects must have been sufficiently healthy to perform moderate exercise as judged by a medical examination including vital signs (ie, resting heart rate, blood pressure, respiration) and an electrocardiogram (ECG). Subjects were required to suspend all current prescription or over-the-counter pain relief medications (ie, nonsteroidal anti-inflammatory drugs [NSAIDs], analgesics, opioids, anti-depressants prescribed for painful conditions such as fibromyalgia, or joint supplements) at least 30 days prior in order to participate in the study. Subjects who were currently taking analgesic or related medications were eligible to participate in the study following a 14-day washout period for NSAIDs, a 7-day washout period for opioids, and a 90-day washout period for injected steroids or antidepressants. Subjects currently taking joint supplements such as glucosamine, chondroitin sulfate, curcumin, and Boswellia were only eligible after a 3-month washout period. Subjects could not meet any of the classification criteria (other than age > 50 years) according to the American College of Rheumatology for

either OA³³ or RA.³⁴ Subjects were also excluded if they were currently receiving remission-inducing drugs such as methotrexate or immunosuppressive medications or had received them within the past 3 months. They were further excluded if they had a confounding inflammatory disease or condition (gout, pseudo gout, lupus, Paget's disease, chronic pain syndrome, etc) that would interfere with assessment of lower-extremity joints. Moreover, subjects were excluded if they participated in activities involving intensive use of the lower extremities (ie, running/jogging, sports, bicycling, dancing, etc) two or more days per week or participated in activities that involved moderate use of the lower extremities (ie, walking, golfing, yoga, etc) three or more days per week. Other exclusionary criteria were body weight 275 pounds (125 kg) or greater, a known allergy to eggs or egg products, or pregnant or breastfeeding women. Subjects previously enrolled in a research study involving an investigational product (drug, device, or biologic) or a new application of an approved product, within 30 days of screening, were also excluded from participating in the trial.

Clinical endpoints/treatment response

The primary endpoint for the study was any statistically significant reduction in exercise-induced cartilage turnover (via the CTX-II biomarker) versus placebo evaluated at 1 and 2 weeks of treatment. Treatment response (reduction) was defined as either a lesser magnitude increase or a decrease from baseline in mean uCTX-II (corrected for creatinine [Cr]) induced by exercise in the NEM group compared to the placebo group. Urine samples were collected from the second void of the morning within 24 hours of completing the final exercise period for each week. Secondary endpoints were any statistically significant reductions in either exercise-induced joint pain or stiffness versus placebo evaluated daily via participant questionnaire. The questionnaire consisted of an immediate assessment (during or up to 1 hour post exercise) and a 12-hour assessment. Each assessment asked the participants to rate their joint pain and stiffness on a 10-point ordinal scale with zero equating to no pain (or stiffness) and 10 equating to severe pain (or stiffness). A similar 10-point ordinal scale was also used to evaluate participant joint pain and stiffness at rest (average from the prior 7 days) and during screening assessment, deemed baseline.

Assessment of uCTX-II

Urine samples were collected from the second void of the morning. Fifteen milliliters from each urine specimen were centrifuged at 3,000 rpm for 10 minutes at 12°C–25°C.

Following centrifuging, two 5 mL aliquots were removed by pipette and were immediately placed in a –20°C freezer. Samples were stored frozen (–20°C) until analysis. Only a single aliquot of the pair was subsequently thawed for initial analysis, to avoid repeated freeze/thaw cycles that might result in aberrant repeat assay values. Urinary concentrations of CTX-II were measured via enzyme-linked immunosorbent assay using a commercial immunoassay (Urine CartiLaps EIA; Immunodiagnostic Systems, Inc, Gaithersburg, MD, USA) and urinary Cr was measured via a colorimetric assay (Cr (urinary) Colorimetric Assay Kit; Cayman Chemical Company, Inc, Ann Arbor, MI, USA) according to the manufacturers' instructions using a SpectraMax Plus 384 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). uCTX-II concentrations were subsequently normalized by dividing uCTX-II expressed in micrograms per liter (µg/L) by urinary Cr expressed in millimoles per liter (mmol/L), the results of which were reported as nanograms of CTX-II per millimole of creatinine (ng/mmol Cr). Samples were assayed in duplicate and all assays were repeated (n=4). Duplicate assay values that were within 90% agreement were subsequently averaged. Repeated assay means that were within 90% agreement were further averaged. If repeat assay means differed by more than 10%, an additional assay was performed in duplicate with the remaining frozen aliquot of urine. The additional repeat assay mean was either substituted for the original outlier, or if insufficient agreement was reached with either original repeat assay mean, then all three were averaged. About 36% of the uCTX-II assays were repeated three times, primarily due to interassay variability. Overall intra-assay coefficients of variation were 6.83 and 2.03 for uCTX-II and Cr, respectively.

Investigational product

The investigational product, NEM, is derived from ESM and is manufactured via a patented process. During the manufacture of NEM, the ESM is partially hydrolyzed utilizing a gentle enzymatic process, as opposed to using harsh indiscriminate chemicals, to enhance gastrointestinal absorption while preserving the membrane's natural biological activity. For this clinical study, 500 mg of NEM (Lot #8012980) or placebo (500 mg of excipients) was provided in #0 vegetarian capsules by ESM Technologies, LLC (Carthage, MO, USA). Treatment and placebo capsules were identical in appearance, odor, and taste and were stored in closed containers at ambient temperature. Participants were instructed to take one capsule daily with water, approximately the same time each morning before eating breakfast.

Safety/adverse events (AEs)

Secondary objectives of the study were to evaluate tolerability and safety or any adverse reactions associated with ingestion of NEM. Subjects underwent a thorough medical examination at screening and upon study completion by a licensed physician including a complete medical history, physical examination, vital signs, ECG, clinical chemistry, hematology, and urinalysis. Vital signs included resting heart rate, blood pressure, respirations, and temperature. Clinical chemistry included alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, protein (total), bilirubin (total), blood urea nitrogen, Cr, potassium, sodium, chloride, calcium, and bicarbonate. Hematology included complete blood count consisting of red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, and white blood cell count with differential (neutrophils, eosinophils, basophils, lymphocytes, and monocytes). Urinalysis included bilirubin, blood, glucose, ketones, nitrites, pH, protein, specific gravity, urobilinogen, and leukocyte esterase. Additionally, participants' self-assessment diaries were reviewed at each clinic visit and any discomfort beyond what would be expected with exercise or any other AEs were recorded and reported in accordance with applicable FDA regulations. AEs and serious AEs were assessed by the clinical investigator and were followed until resolution, as necessary. Serious AEs were required to be reported to the clinical monitor immediately.

Statistical analysis

The hypothesis for this study was that the treatment group would be superior to that of the placebo group in limiting the increase in uCTX-II levels resulting from moderate exercise. A 16% absolute change in the mean treatment response (uCTX-II would increase by an average of at least 16% more in the placebo group than in the treatment group) was expected based upon the prior proof-of-concept trial (unpublished). We estimated that a sample size of 60 subjects would need to be enrolled to provide the study with a statistical power of 80% to detect a clinically meaningful difference between the treatment group and the placebo group, assuming a rate of response of -4% for the treatment group and a rate of response of $+12\%$ for the placebo group, with no dropouts. As the actual enrollment for the study was 60 subjects, this should be sufficient to provide adequate safety and comparative effectiveness information. Descriptive statistics were calculated including mean age, height, weight, BMI, and number of steps per leg, and comparisons of this demographic

data were made with a Kruskal–Wallis test for multiple independent samples at baseline to validate randomization. Following evaluation for normality, postbaseline statistical analyses were done utilizing either an independent-group *t*-test (CTX-II) or repeated-measures univariate analysis of variance (rm-ANOVA) (pain and stiffness). Items found to have statistical significance with rm-ANOVA were then compared using a Kruskal–Wallis test for multiple independent samples. In all cases, statistical significance was accepted at $P < 0.05$. Analysis of the primary endpoint, as well as all secondary endpoints, was conducted on the per-protocol population. SYSTAT software (version 13) (Systat Software, Inc, San Jose, CA, USA) was used for all statistical analyses.

Results

Recruitment began in November 2015 at a single clinical site in Missouri and the final follow-up was conducted in January 2016. One hundred seventy-two (female) subjects were screened and a total of 60 (female) subjects between the ages of 44 and 74 years were enrolled in the trial and underwent randomization. Thirty subjects (50.0%) were randomized to the placebo group and 30 subjects (50.0%) were randomized to the NEM treatment group. All subjects completed the study per the protocol and there were no dropouts (0%). Compliance with the study treatment regimen was good in both treatment groups, as judged by capsule count at clinic visits and all subjects attended every assigned exercise period.

Participant demographic data (Table 1) was initially evaluated to validate randomization. There were no statistical

Table 1 Participant baseline demographic data

Demographic parameters	NEM	Placebo
Age, years	56.1±7.4	57.7±5.9
Sex		
Female (%)	30 (100)	30 (100)
Height, cm	164±5	164±7
Weight, kg	75.7±13.6	78.1±14.5
BMI, kg/m ²	28.2±5.4	28.8±4.4
uCTX-II, ng/mmol Cr	258±106	236±175
Resting pain score	0.2±0.4	0.5±0.8
Resting stiffness score	0.3±0.6	0.6±0.9
Number of steps per leg	61.9±16.2	59.3±11.9
Baseline pain score	1.4±1.1	1.4±1.4
Baseline stiffness score	1.3±1.0	1.7±1.3

Notes: Except where indicated otherwise, values are reported as mean ± SD (n=30 per group). There were no statistical differences between treatment groups in any of the listed parameters. Baseline pain and stiffness scores were obtained from performing the number of steps per leg determined at screening.

Abbreviations: BMI, body mass index, calculated as weight in kilograms divided by the square of height in meters; NEM, natural eggshell membrane; uCTX-II, urinary C-terminal cross-linked telopeptide of type-II collagen, reported as nanograms per millimole of creatinine (ng/mmol Cr).

differences between treatment groups in any of the parameters listed (not shown). Importantly, parameters expected to have a bearing on endpoint differences (ie, BMI, steps per leg, uCTX-II, baseline pain, and stiffness) were evenly distributed between treatment groups. A clinical comparison of valid subjects was carried out to obtain mean scores for each of the outcome measures (CTX-II, pain, and stiffness) after 1 week and 2 weeks of exercise (Table 2). Absolute treatment effects (TE_{abs}) for all outcome measures were calculated as the net difference of treatment versus placebo for the mean change in treatment effect from baseline (or resting) for each group expressed as a percent. Negative values represent superior results in the treatment group. Statistical analysis of the primary outcome measure (CTX-II) revealed that supplementation with NEM produced a significant treatment response versus placebo after both 1 week (TE_{abs} -17.2%, $P=0.002$) and 2 weeks of exercise (TE_{abs} -9.9%, $P=0.042$) (Figure 1). The overall trend for immediate pain was not significantly different from placebo ($P=0.209$) (Figure 2A), despite a statistically significant decrease from baseline for Days 7–13 in the NEM treatment group (Day 13 TE_{abs} -38.1%), whereas the placebo group was not significantly different from baseline at any time point. The overall trend for recovery (12-hour) pain was significantly different from placebo ($P=0.016$), and this difference occurred at Day 8 and continued through Day 14 (end of

study) (Figure 2B). By the end of the 2-week evaluation period, recovery pain had nearly returned to resting levels for the NEM treatment group (Day 14 TE_{abs} -11.8%), while placebo group recovery pain levels remained substantially elevated. The overall trend for immediate stiffness was significantly different from placebo ($P=0.042$) and this difference occurred at Days 7 and 11, with Days 3, 5, 9, and 13 showing a positive trend ($P<0.10$) for being different from placebo (Figure 3A). Both groups experienced less immediate stiffness from performing the exercise regimen as the study progressed; however, the NEM treatment group felt a greater benefit (Day 13 TE_{abs} -18.5%). The overall trend for recovery (12-hour) stiffness was also significantly different from placebo ($P=0.014$) and this difference occurred at Days 4, 8, 12, and 14, with Days 2, 6, and 10 showing a positive trend ($P<0.10$) for being different from placebo (Figure 3B). Similar to recovery pain, recovery stiffness had nearly returned to resting levels for the NEM treatment group (Day 14 TE_{abs} -56.3%), while the placebo group recovery stiffness levels remained substantially elevated.

There were 25 non-exercise-related AEs reported by the treatment group, the most common of which were eight instances of headache, three instances of nausea, and three instances of cold/flu/sinus congestion. None of the treatment group AEs were judged by the clinical investigator to be associated with treatment. There were 32 non-exercise-related AEs reported by the placebo group, the most common of which were 12 instances of headache, three instances of nausea, and five instances of cold/flu/sinus congestion. None of the placebo group AEs were judged by the clinical investigator to be associated with treatment. There were no serious AEs reported during the study in either treatment group. Although there were a greater number of AEs in the placebo group (56%) versus the NEM group (44%), the overall use of acetaminophen was similar between the groups. There were seven instances of acetaminophen use in the placebo group (avg 18.6 mg/subject/day) and eight instances in the NEM treatment group (avg 18.0 mg/subject/day) with the vast majority of these instances being related to either headache or cold/flu/sinus congestion. The treatment was reported to be well tolerated by study participants.

Discussion

Discomfort during and resulting from exercise is extremely common, particularly for those 45 years and older. It is important to minimize this discomfort and to mitigate any long-term consequences that may result in order to realize the myriad health benefits of regular exercise, and in the elderly

Table 2 Mean scores for CTX-II and (immediate and 12-hour) pain and stiffness in NEM-supplemented and placebo groups at baseline (or resting) and after 1 and 2 weeks of exercise

Parameter	Weeks	Treatment	
	Post treatment	NEM	Placebo
uCTX-II	Resting (n=30, 30)	258±106	236±175
	1 (n=30, 30)	234±83*	255±172*
	2 (n=30, 30)	242±101#	245±184
Immediate pain	Baseline (n=30, 30)	1.4±1.1	1.4±1.4
	1 (n=30, 30)	1.0±1.1*	1.6±1.5
	2 (n=30, 30)	0.7±0.9*	1.3±1.5
12-hour pain	Resting (n=30, 30)	0.2±0.4	0.5±0.8
	1 (n=30, 30)	0.4±0.6*	1.0±1.3*
	2 (n=30, 30)	0.3±0.6#	0.8±1.2#
Immediate stiffness	Baseline (n=30, 30)	1.3±1.0	1.7±1.3
	1 (n=30, 30)	0.9±0.9#	1.7±1.5
	2 (n=30, 30)	0.7±0.9*	1.2±1.5#
12-hour stiffness	Resting (n=30, 30)	0.3±0.6	0.6±0.9
	1 (n=30, 30)	0.5±0.7*	1.0±1.2*
	2 (n=30, 30)	0.3±0.6	0.9±1.4#

Notes: Values are reported as mean ± SD. * $P<0.05$; # $P<0.10$ within group from baseline or resting.

Abbreviations: NEM, natural eggshell membrane; uCTX-II, urinary C-terminal cross-linked telopeptide of type-II collagen, reported as nanograms per millimole of creatinine (ng/mmol Cr).

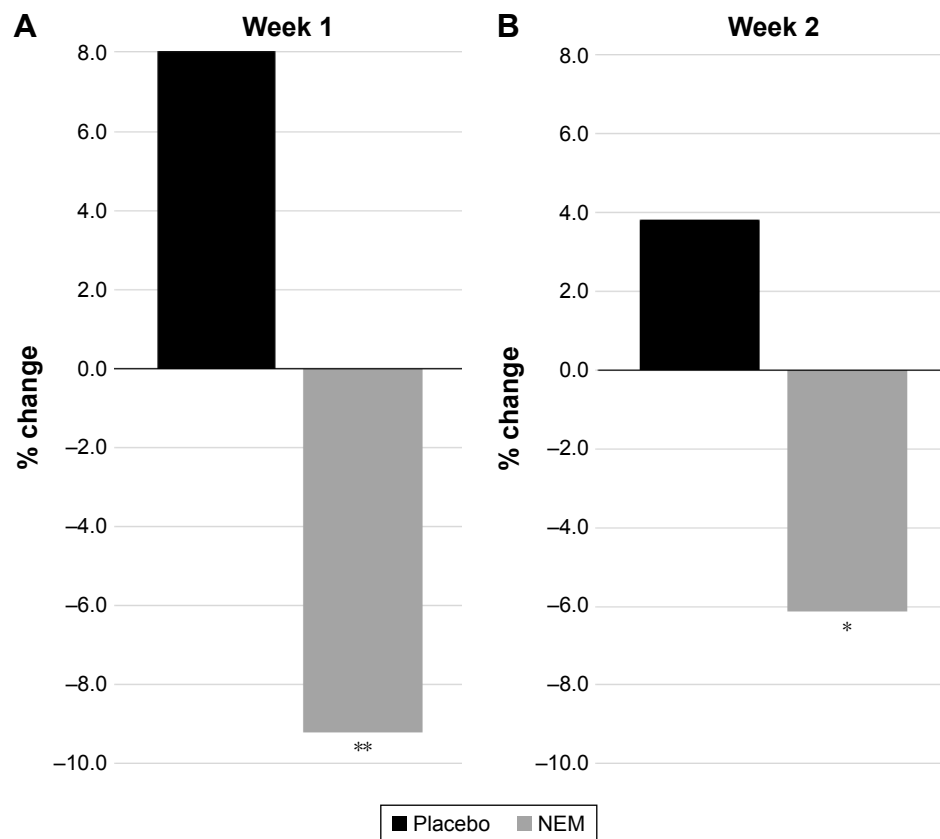


Figure 1 Percent change from resting in urinary CTX-II in NEM-supplemented and placebo groups after 1 week (A) and 2 weeks (B) of exercise.

Notes: * $P < 0.05$; ** $P < 0.01$ versus placebo.

Abbreviations: CTX-II, C-terminal cross-linked telopeptide of type-II collagen, reported as nanograms per millimole of creatinine (ng/mmol Cr); NEM, natural eggshell membrane.

to prevent the detrimental musculoskeletal changes that are a consequence of immobility. This trial was designed to evaluate the effects of NEM brand ESM in relieving exercise-induced pain, stiffness, and cartilage turnover in healthy, postmenopausal women. NEM demonstrated meaningful beneficial effects for all three of these clinical endpoints.

NEM-treated subjects experienced 38.1% less exercise-induced (immediate) pain than placebo subjects by the end of the 2-week evaluation period. Although this difference failed to reach statistical significance in the overall trend, Day 11 differed statistically from placebo and Day 13 fell just shy of significance. This substantial decrease from baseline for the NEM-treated group was also statistically significant (within group) for Days 7–13, whereas it was not different from baseline at any time point for the placebo group. Combined, these two statistical comparisons suggest that the overall trend may have reached significance had the study had slightly greater enrollment or had the evaluation period continued for an additional few days to a week. The overall trend for recovery from exercise-induced pain was statistically significant for NEM-treated subjects compared

to the placebo group. This improved pain recovery occurred just after 1 week (Day 8) of supplementation with NEM and persisted through the remainder of the study. Importantly, recovery pain had nearly returned to resting levels for the NEM-treated subjects by the end of the 2-week evaluation period while placebo group recovery pain levels remained substantially elevated.

NEM-treated subjects experienced 18.5% less exercise-induced (immediate) stiffness and recovered 56.3% better than placebo subjects by the end of the 2-week evaluation period. Both of these substantial improvements were statistically different from placebo overall, with the difference in immediate stiffness occurring after 1 week (Day 7) of supplementation with NEM and recovery from stiffness differing after only 4 days of supplementation. While these differences were not continuously significant across the entire evaluation period, the discontinuous timepoints fell just shy of significance ($P < 0.10$). Again, this issue likely would have been resolved had enrollment been slightly greater. Similar to recovery pain, recovery stiffness had nearly returned to resting levels for the NEM-treated group

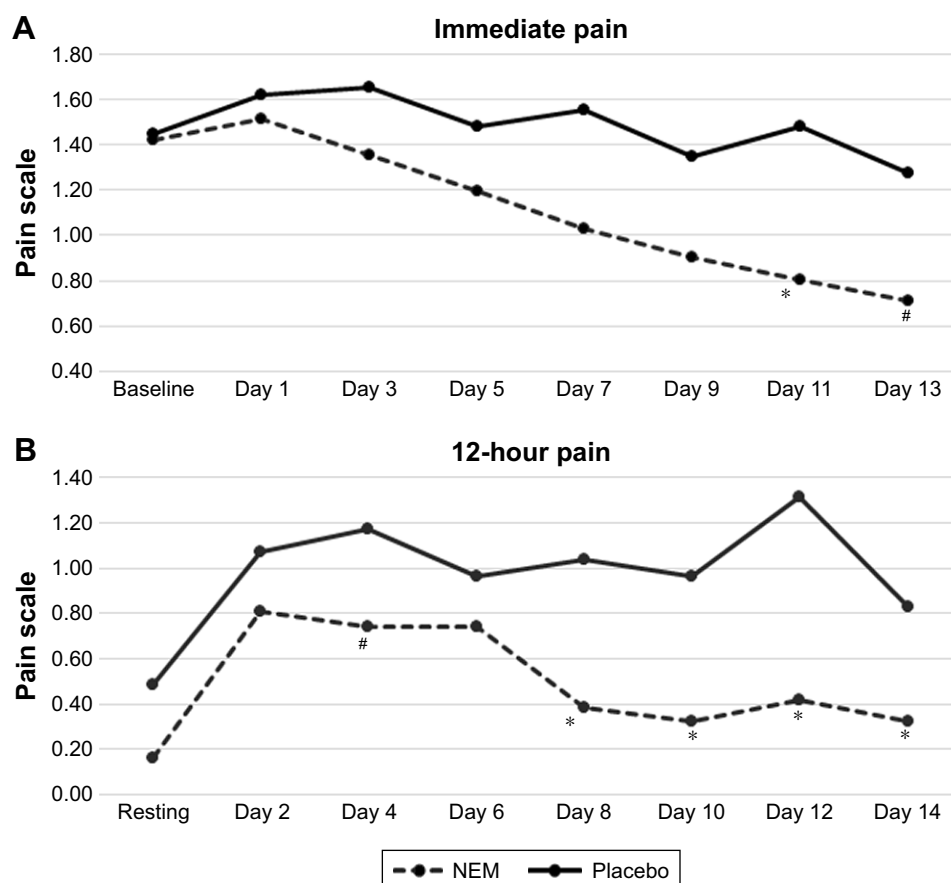


Figure 2 A plot of immediate pain (A) and 12-hour pain (B) in NEM-supplemented and placebo groups across 2 weeks of exercise.

Notes: Overall trend differences were determined via rm-ANOVA. Trend differences found to have statistical significance with rm-ANOVA were then compared using a Kruskal–Wallis test for multiple independent samples to identify specific time points that differed between treatment and placebo. * $P < 0.05$; # $P < 0.10$ versus placebo.

Abbreviations: NEM, natural eggshell membrane; rm-ANOVA, repeated-measures analysis of variance.

while placebo group recovery stiffness levels again remained substantially elevated.

Interestingly, recovery from stiffness was more rapid (Day 4 vs Day 8) and exhibited a greater treatment response (–56% vs –12%) than recovery from pain. This is generally consistent with what has been observed in prior clinical studies with NEM that were conducted in OA subjects,^{27–29} particularly in relation to the magnitude of the treatment responses. This observed clinical effect is also consistent with the proposed mechanism of action of NEM. That is, the reduction in proinflammatory cytokines demonstrated in previous mechanistic investigations^{25,26,30} would be expected to have a direct correlation to joint stiffness, which is a likely result of localized inflammation. Although NEM has also been shown to affect pain signal transduction via reducing prostaglandin E_2 (PGE_2) in an OA rat model,³⁰ the corresponding clinical effect would be expected to lag behind any anti-inflammatory effect as PGE_2 synthesis is amplified by the inducible enzyme, cyclooxygenase-2 (COX-2), that is induced by and responsive to proinflammatory cytokines.³⁵

Moreover, the perception of pain (nociception) has been shown to be hypersensitized by proinflammatory cytokines³⁶ wherein normally painful stimuli are perceived as more painful. This hyperalgesia is thought to contribute substantially to the nature of chronic pain perception in joint diseases like OA.³⁷ A hydrolysate of NEM was also found to moderately inhibit the enzyme 5-lipoxygenase (5-LOX) (IC_{50} 11,188 $\mu\text{g/mL}$) in vitro while not inhibiting COX-1 or COX-2 to any appreciable extent (unpublished). Leukotrienes are produced via the 5-LOX conversion of cell-surface arachidonic acid and have been shown to play a significant role in both nociception and chronic and inflammatory pain pathways.³⁸ One of these lipid proinflammatory mediators, leukotriene B_4 , was found to be significantly reduced by NEM in an OA rat model.³⁰

NEM-treated subjects also experienced markedly less cartilage degradation than the placebo group. uCTX-II levels were 17.2% lower after 1 week of supplementation with NEM while this reduction moderated slightly to –9.9% after 2 weeks of supplementation. When the trial was initially

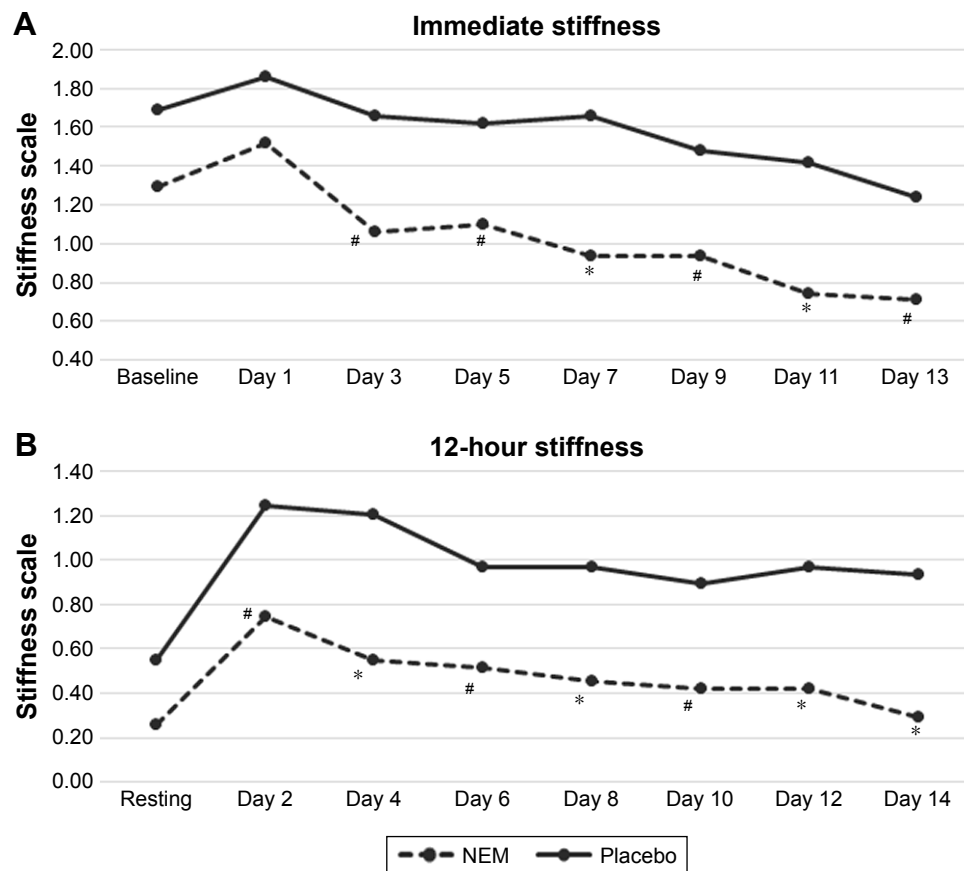


Figure 3 A plot of immediate stiffness (**A**) and 12-hour stiffness (**B**) in NEM-supplemented and placebo groups across 2 weeks of exercise.

Notes: Overall trend differences were determined via rm-ANOVA. Trend differences found to have statistical significance with rm-ANOVA were then compared using a Kruskal-Wallis test for multiple independent samples to identify specific time points that differed between treatment and placebo. * $P < 0.05$; # $P < 0.10$ versus placebo.

Abbreviations: NEM, natural eggshell membrane; rm-ANOVA, repeated-measures analysis of variance.

conceived, it was expected that NEM would simply mitigate the increase in urinary outflux of CTX-II resulting from exercise-induced cartilage degradation. It is noteworthy that NEM not only prevented such an increase, but actually reduced uCTX-II below baseline levels (Figure 1). This remarkable chondroprotective effect resulting from treatment with a natural health product is the first instance, to our knowledge, demonstrated in healthy subjects. Similarly, a profound chondroprotective effect from NEM treatment was recently demonstrated for the first time in dogs with naturally occurring joint disease.³² NEM was initially shown to affect CTX-II in a rat model of OA³⁰ wherein this effect was found to correlate with chondroprotection via histopathology and micro-CT-arthrography. In that study, NEM treatment suppressed cartilage deformation and preserved cartilage volume compared to untreated OA controls. At least part of this chondroprotection may result from the anti-inflammatory properties of NEM noted previously; however, serum levels of matrix metalloproteinases (MMP-2 and -9) known to degrade cartilage, were also substantially reduced.³⁰

NEM's effect on CTX-II production may be further evidence of a second immunomodulatory mechanism of action outside of the typical direct interaction with immune cells in the blood or inflamed tissues. That is, 50% of OA patients were found to have autoantibodies to type II collagen³⁹ and these autologous fragments as well as other cartilage-derived fragments are thought to be a major driver of the immune-mediated cartilage destruction that typifies OA progression. The immune component of OA includes antigen-specific proliferation of T cells, synovial membrane infiltration, and the subsequent localized production of proinflammatory cytokines including IL-1 β and TNF- α among others.³⁹ Similarly, collagen-induced arthritis (CIA), a well-accepted animal model of RA, is initiated by subdural injection of type II collagen in combination with an immunogenic adjuvant. The adjuvant functions via the activation of the cytoplasmic transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the subsequent antigen-specific T-cell proliferation pathway that closely mirrors that described above for OA.

This CIA rat model has been used to validate an immune regulatory process known as “oral tolerance”. Oral tolerance refers to the phenomenon of a reduced peripheral immune response (tolerance) that results from the repeated exposure of the mucosal immune system in the gut to ingested protein antigens. Oral tolerance to immunogenic peptides that are repeatedly ingested is believed to result from immune surveillance within the gut-associated lymphoid tissue as a way for the body to prevent an inappropriate or unnecessary immune response to proteins normally consumed in the diet. A hydrolysate of NEM was found to activate NF- κ B in vitro,⁴⁰ and in a subsequent study employing the CIA rat model, oral supplementation with NEM substantially suppressed swelling due to inflammation and markedly lessened cartilage damage, pannus formation, and periarticular bone resorption histologically.³¹ Immune tolerance to cartilage components, particularly type II collagen, from oral supplementation with NEM (which contains types I, V, and X collagen) is a more likely explanation for the longer-term reduction in CTX-II formation demonstrated here than is direct suppression of localized inflammation in the joints experiencing strain from exercise.

There was a moderate number of AEs in the trial, all of which were deemed unrelated to treatment, and no serious AEs occurred. Although there were a greater number of AEs in the placebo group (56%) versus the NEM group (44%), the overall use of acetaminophen was similar between the groups with seven instances and eight instances, respectively, with the vast majority of these instances being related to either headache or cold/flu/sinus congestion. No side effects were noted in this trial, nor in any of the five prior trials published to date.^{27–29} Food-derived natural products such as NEM would be expected to have a robust safety profile, and this has been confirmed throughout its clinical research experience, excluding the obvious egg allergy concern.

Conclusion

NEM brand ESM, 500 mg once daily, rapidly improved recovery from exercise-induced joint pain (Day 8) and stiffness (Day 4) and also significantly reduced the discomfort from stiffness immediately following exercise (Day 7). Moreover, a substantial chondroprotective effect was demonstrated from supplementation with NEM through a lasting decrease in the cartilage degradation biomarker CTX-II. There were no dropouts in the study, and treatment with NEM was reported to be well tolerated. The beneficial effects of NEM versus placebo in exercise-induced joint pain, stiffness, and cartilage turnover described here for the first time in healthy,

postmenopausal women should help women of this age group to stay active and maintain healthy joints as they age. The ability to recover quickly from exercise-induced discomfort (both pain and stiffness) could lead to more frequent exercise and may ultimately reduce the rate of exercise discontinuation. And although not a sensory response, the knowledge that one’s joint cartilage is also being protected from damage due to exercise should further improve one’s inclination to exercise and continue exercising.

Acknowledgment

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Disclosure

KJR and MB are employees of ESM Technologies, LLC. CA is an independent distributor for ESM’s product. JT is a paid consultant for ESM Technologies, LLC. The authors report no other conflicts of interest in this work.

References

- Haskell WL, Lee IM, Pate RR, et al. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Circulation*. 2007;116(9):1081–1093.
- Sofia F, Capalbo A, Cesari F, Abbate R, Gensini GF. Physical activity during leisure time and primary prevention of coronary heart disease: an updated meta-analysis of cohort studies. *Eur J Cardiovasc Prev Rehabil*. 2008;15(3):247–257.
- Sigal RJ, Kenny GP, Wasserman DH, Castaneda-Sceppa C, White RD. Physical activity/exercise and type 2 diabetes. *Diabetes Care*. 2006; 29(6):1433–1438.
- Warburton DE, Nicol CW, Bredin SS. Health benefits of physical activity: the evidence. *Can Med Assoc J*. 2006;174(6):801–809.
- Nelson ME, Rejeski WJ, Blair SN, et al. Physical activity and public health in older adults: recommendation from the American College of Sports Medicine and the American Heart Association. *Circulation*. 2007;116(9):1094–1105.
- Ensrud KE, Ewing SK, Cawthon PM, et al. A comparison of frailty indexes for the prediction of falls, disability, fractures, and mortality in older men. *J Am Geriatr Soc*. 2009;57(3):492–498.
- Ensrud KE, Ewing SK, Taylor BC, et al. Comparison of 2 frailty indexes for prediction of falls, disability, fractures, and death in older women. *Arch Intern Med*. 2008;168(4):382–389.
- Pisters MF, Veenhof C, van Meeteren NL, et al. Long-term effectiveness of exercise therapy in patients with osteoarthritis of the hip or knee: a systematic review. *Arthritis Rheum*. 2007;57(7):1245–1253.
- Hochberg MC, Altman RD, April KT, et al. American College of Rheumatology 2012 recommendations for the use of nonpharmacologic and pharmacologic therapies in osteoarthritis of the hand, hip, and knee. *Arthritis Care Res (Hoboken)*. 2012;64(4):465–474.
- Valderrabano V, Steiger C. Treatment and prevention of osteoarthritis through exercise and sports. *J Aging Res*. 2010;2011:374653.
- Akeson WH, Amiel D, Abel MF, Garfin SR, Woo SL. Effects of immobilization on joints. *Clin Orthop Relat Res*. 1987;219:28–37.
- Brandt KD. Response of joint structures to inactivity and to reloading after immobilization. *Arthritis Rheum*. 2003;49(2):267–271.
- Lotz M, Martel-Pelletier J, Christiansen C, et al. Value of biomarkers in osteoarthritis: current status perspectives. *Ann Rheum Dis*. 2013; 72(11):1756–1763.

14. Valdes AM, Meulenbelt I, Chassaing E, et al. Large scale meta-analysis of urinary C-terminal telopeptide, serum cartilage oligomeric protein and matrix metalloprotease degraded type II collagen and their role in prevalence, incidence and progression of osteoarthritis. *Osteoarthritis Cartilage*. 2014;22(5):683–689.
15. van Spil WE, DeGroot J, Lems WF, Oostveen JC, Lafeber FP. Serum and urinary biochemical markers for knee and hip-osteoarthritis: a systematic review applying the consensus BIPED criteria. *Osteoarthritis Cartilage*. 2010;18(5):605–612.
16. Sowers MF, Karvonen-Gutierrez CA, Yosefy M, et al. Longitudinal changes of serum COMP and urinary CTX-II predict X-ray defined knee osteoarthritis severity and stiffness in women. *Osteoarthritis Cartilage*. 2009;17(12):1609–1614.
17. Sharif M, Kirwan J, Charni N, Sandell LJ, Whittles C, Garner P. A 5-year longitudinal study of type IIA collagen synthesis and total type II collagen degradation in patients with knee osteoarthritis-association with disease progression. *Rheumatol (Oxford)*. 2007;46(6):938–943.
18. Reijman M, Hazes JM, Bierma-Zeinstra SM, et al. A new marker for osteoarthritis: cross-sectional and longitudinal approach. *Arthritis Rheum*. 2004;50(8):2471–2478.
19. Dam EB, Byrjalsen I, Karsdal MA, Qvist P, Christiansen C. Increased urinary excretion of C-telopeptides of type II collagen (CTX-II) predicts cartilage loss over 21 months by MRI. *Osteoarthritis Cartilage*. 2009;17(3):384–389.
20. Eyre DR, Shao P, Vosberg-Smith K, Weis M, Shaffer K, Yoshihara P. Cross-linked telopeptides from collagen types I, II and III in human urine. *J Bone Miner Res*. 1996;11(S1):S413.
21. O’Kane JW, Hutchinson E, Atley LM, Eyre DR. Sport-related differences in biomarkers of bone resorption and cartilage degradation in endurance athletes. *Osteoarthritis Cartilage*. 2006;14(1):71–76.
22. Mouritzen U, Christgau S, Lehmann HJ, Tankó LB, Christiansen C. Cartilage turnover assessed with a newly developed assay measuring collagen type II degradation products: influence of age, sex, menopause, hormone replacement therapy, and body mass index. *Ann Rheum Dis*. 2003;62(4):332–336.
23. Wong M, Hendrix MJ, von der Mark K, Little C, Stern R. Collagen in the egg shell membranes of the hen. *Dev Biol*. 1984;104(1):28–36.
24. Baker JR, Balch DA. A study of the organic material of hen’s-egg shell. *Biochem J*. 1962;82:352–361.
25. Benson KF, Ruff KJ, Jensen GS. Effects of Natural Eggshell Membrane (NEM) on cytokine production in cultures of peripheral blood mononuclear cells: increased suppression of tumor necrosis factor- α levels after in vitro digestion. *J Med Food*. 2012;15(4):360–368.
26. Ruff KJ, DeVore DP. Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Mod Res Inflamm*. 2014;3(1):19–25.
27. Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW. Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin Rheumatol*. 2009;28(8):907–914.
28. Danesch U, Seybold M, Rittinghausen R, Treibel W, Bitterlich N. NEM® brand eggshell membrane effective in the treatment of pain associated with knee and hip osteoarthritis: results from a six-center, open-label German Clinical Study. *J Arthritis*. 2014;3(3):136.
29. Brunello E, Masini A. NEM® brand eggshell membrane effective in the treatment of pain and stiffness associated with osteoarthritis of the knee in an Italian Study Population. *Int J Clin Med*. 2016;7(1):169–175.
30. Sim BY, Bak JW, Lee HJ, et al. Effects of natural eggshell membrane (NEM) on monosodium iodoacetate-induced arthritis in rats. *J Nutr Health*. 2015;48(4):310–318.
31. Wedekind KJ, Ruff KJ, Atwell CA, Evans JL, Bendele AM. Beneficial effects of natural eggshell membrane (NEM) on multiple indices of arthritis in collagen-induced arthritic rats. *Modern Rheumatol*. 2017;27(5):838–848.
32. Ruff KJ, Kopp KJ, Von Behrens P, Lux M, Mahn M, Back M. Effectiveness of NEM® brand eggshell membrane in the treatment of sub-optimal joint function in dogs: a multi-center, randomized, double-blind, placebo-controlled study. *Vet Med Res Rep*. 2016;7:113–121.
33. Altman R, Asch E, Bloch D, et al. Development of criteria for the classification and reporting of osteoarthritis: osteoarthritis of the knee. *Arthritis Rheum*. 1986;29(8):1039–1049.
34. Aletaha D, Neogi T, Silman AJ, et al. 2010 Rheumatoid arthritis classification criteria – an American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum*. 2010;62(9):2569–2581.
35. Kawabata A. Lipid mediators and pain signaling: prostaglandin E2 and pain – an update. *Biol Pharm Bull*. 2011;34(8):1170–1173.
36. Kress M. Nociceptor sensitization by proinflammatory cytokines and chemokines. *Open Pain J*. 2010;3:97–107.
37. Schiabel HG. Mechanisms of chronic pain in osteoarthritis. *Curr Rheumatol Rep*. 2012;14(6):549–556.
38. Noguchi K, Okubo M. Lipid mediators and pain signaling: leukotrienes in nociceptive pathway and neuropathic/inflammatory pain. *Biol Pharm Bull*. 2011;34(8):1163–1169.
39. Sakkas LI, Platsoucas CD. The role of T cells in the pathogenesis of osteoarthritis. *Arthritis Rheum*. 2007;56(2):409–424.
40. Ruff KJ, Durham PL, O’Reilly A, Long FD. Eggshell membrane hydrolyzates activate NF- κ B in vitro: possible implications for in vivo efficacy. *J Inflamm Res*. 2015;8:49–57.

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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_{d0-17}), 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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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

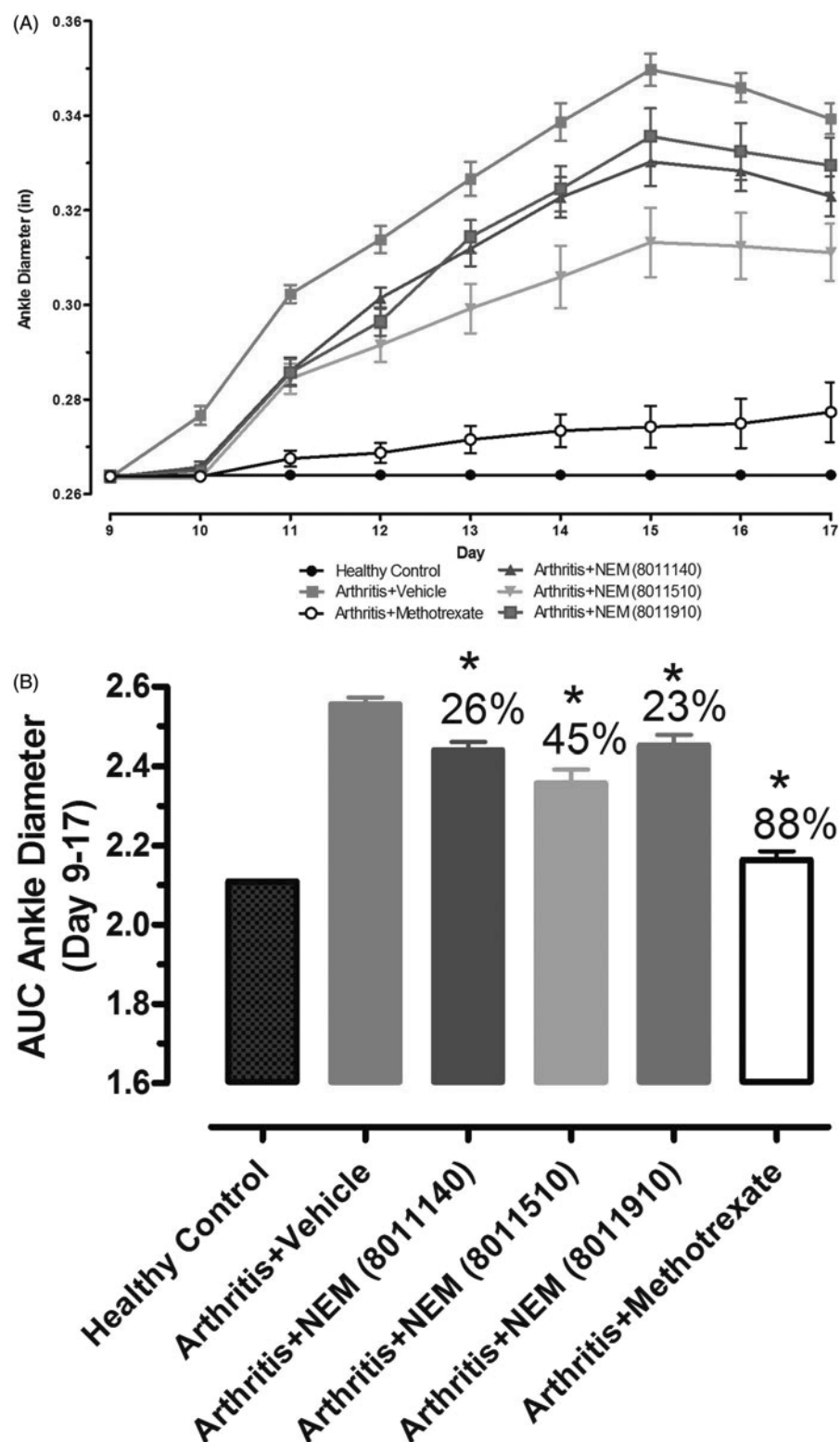


Figure 1. Effect of NEM on ankle diameter. Ankle measurements (Panel a) and AUC (Panel b) calculations were performed as described in Materials and Methods. Numbers above bars in Panel b indicate % inhibition calculated as described in Materials and Methods. Asterisks indicate statistical significance,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

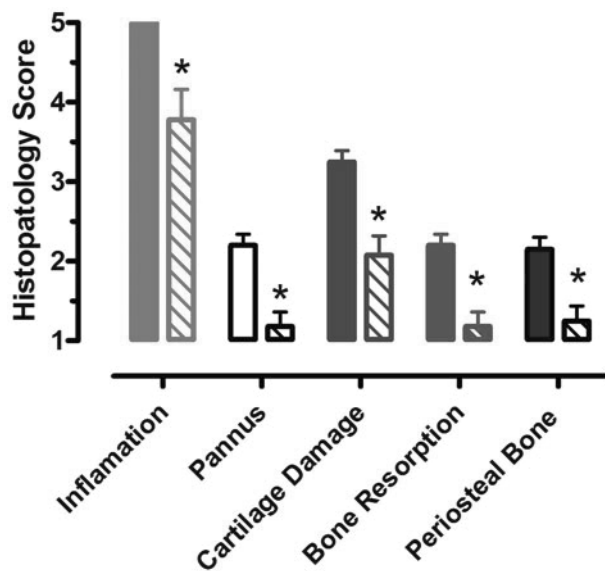


Figure 2. Effects of NEM on individual ankle histopathology scores. Collagen arthritic ankles were given scores of 0–5 for inflammation, pannus formation, cartilage damage, bone resorption, and periosteal new bone formation according to the criteria indicated in Supplementary Information. Vehicle control, 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

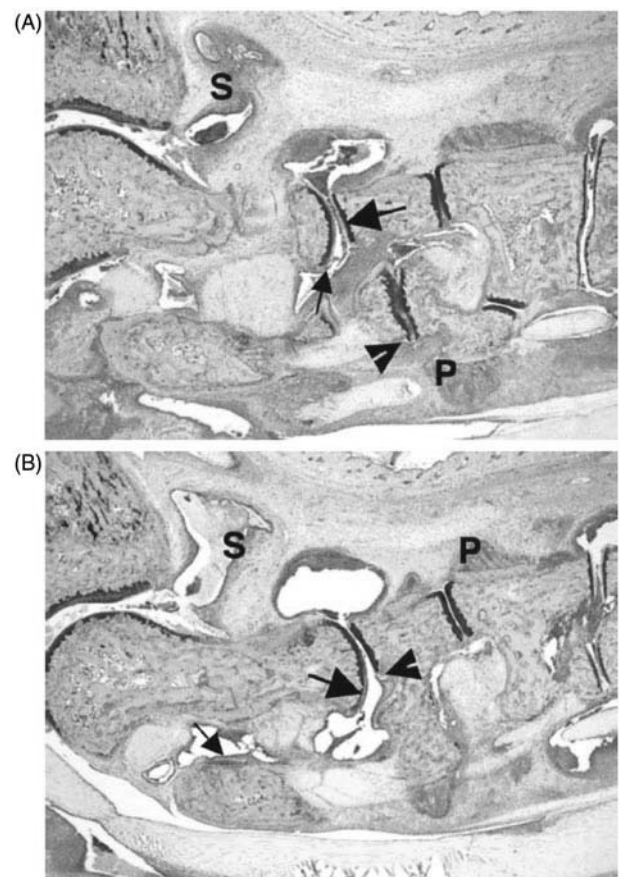


Figure 3. Effects of NEM on morphologic pathology ankle joint. Panel A: Ankle from an arthritis vehicle control animal (with the approximate mean summed score for the group; animal # 7, left ankle) has severe inflammation (S) and moderate cartilage damage (large arrow) with mild pannus (small arrow) 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

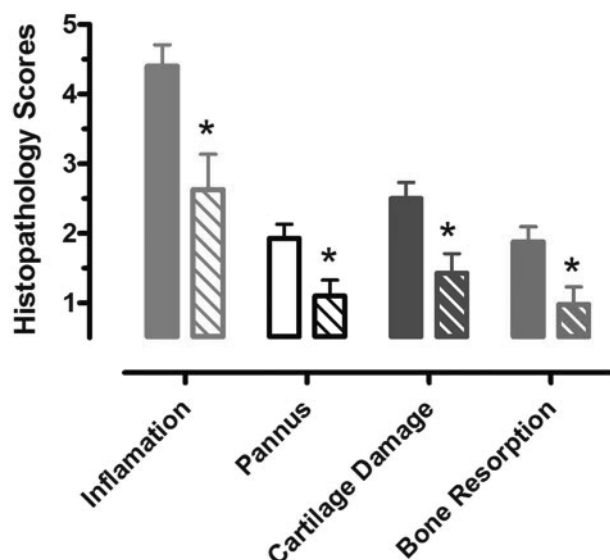


Figure 4. Effects of NEM on individual knee histopathology scores. Collagen arthritic knees were given scores of 0–5 for inflammation, pannus formation, cartilage damage, and bone resorption according to the criteria indicated in Supplementary Information. Vehicle control, solid bars; NEM-8011510, hatched 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

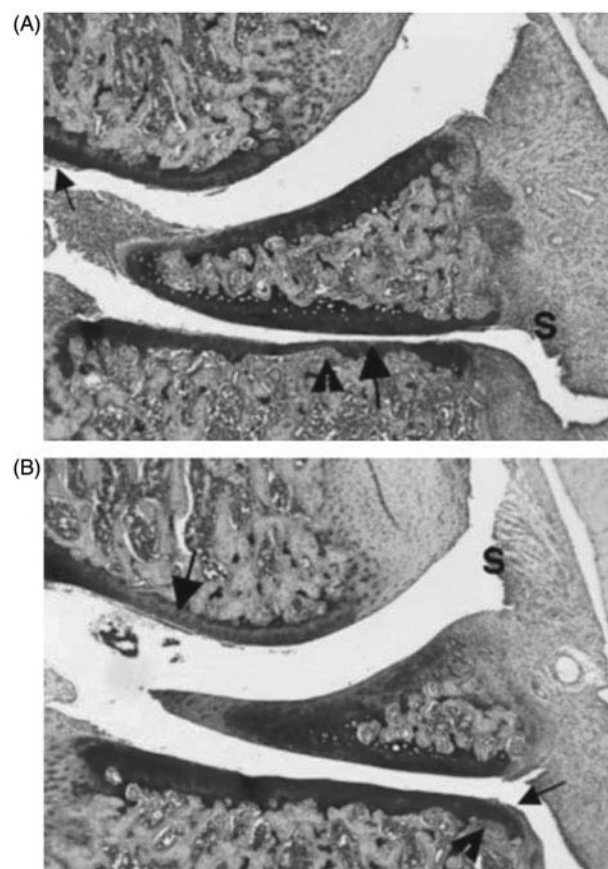


Figure 5. Effects of NEM on morphologic pathology knee joint. Panel A: Knee from a arthritis vehicle control animal (with the approximate mean summed score for the group; animal 1, right knee) has severe inflammation (S) with mild pannus (small arrow), cartilage damage (large arrow), and bone resorption (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

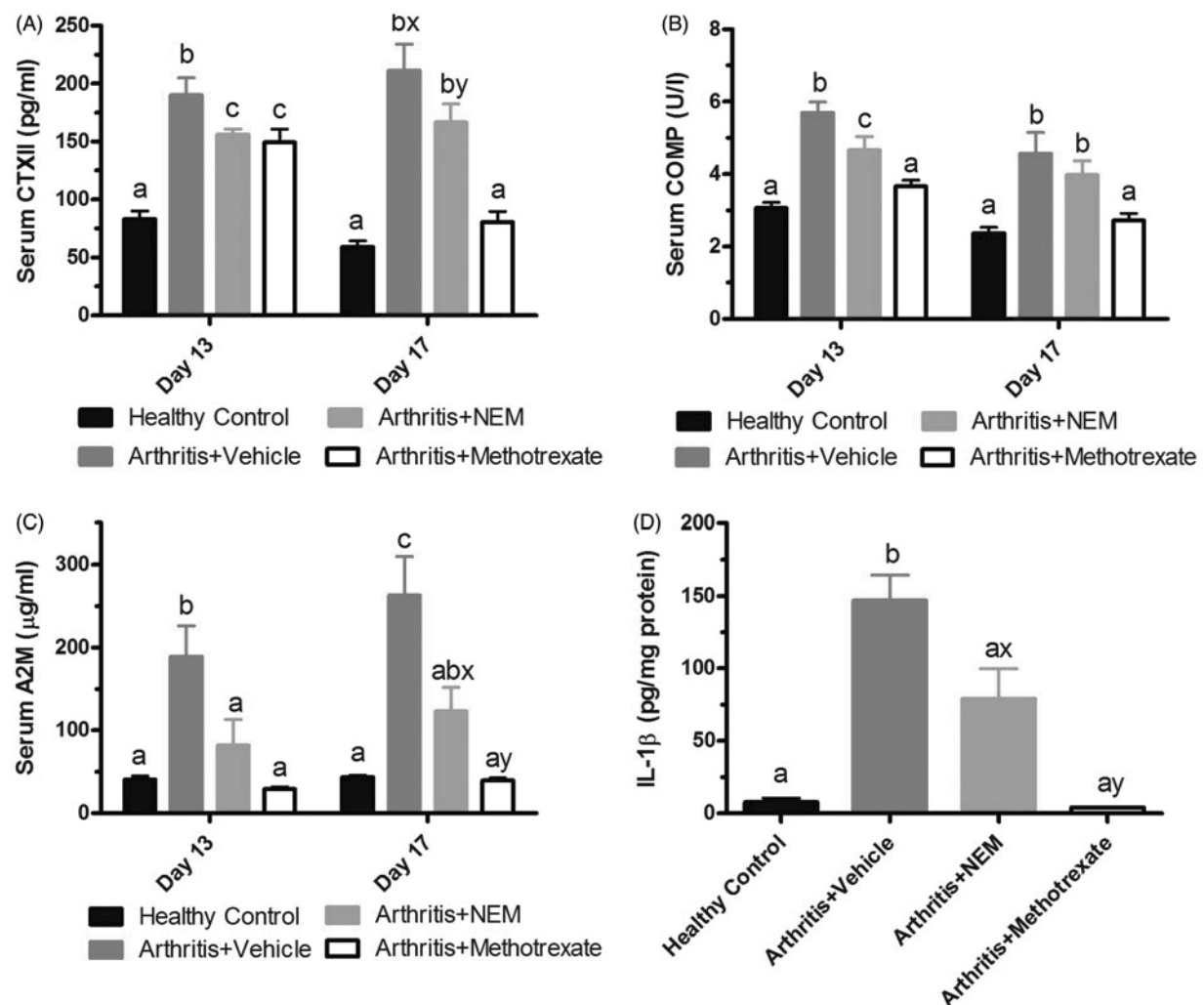


Figure 6. Effects of NEM on biomarkers CTXII, COMP, A2M, and IL-1 β . Biomarkers were measured as described in Materials and Methods. CTX II, COMP, and A2M were measured in serum samples, and IL-1 β was measured in knee lavage fluid. Bars with different letters indicate that they were statistically different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


Acknowledgments

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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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References

1. Erickson AR, Cannella AC, Mikuls TR. Clinical features of rheumatoid arthritis. In: Firestein GS, Budd RC, Gabriel SE, McInnes IB, O'Dell JR, eds. Kelley and Firestein's textbook of rheumatology. 10th ed. Philadelphia: Elsevier; 2016:1167–86.
2. Firestein GS. Etiology and pathogenesis of rheumatoid arthritis. In: Firestein GS, Budd RC, Gabriel SE, McInnes IB, O'Dell JR, eds. Kelley and Firestein's textbook of rheumatology. 10th ed. Philadelphia: Elsevier; 2016:1115–66.e7.
3. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The global burden of rheumatoid arthritis: estimates from the global burden of disease 2010 study. *Ann Rheum Dis*. 2014;73:1316–22.

4. Gibofsky A. Overview of epidemiology, pathophysiology, and diagnosis of rheumatoid arthritis. *Am J Manag Care*. 2012;18: S295–S302.
5. Doran MF, Pond GR, Crowson CS, O'Fallon WM, Gabriel SE. Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period. *Arthritis Rheum*. 2002;46:625–31.
6. Zampeli E, Vlachoyiannopoulos PG, Tzioufas AG. Treatment of rheumatoid arthritis: unraveling the conundrum. *J Autoimmun*. 2015;65:1–18.
7. Finckh A, Bansback N, Marra CA, Anis AH, Michaud K, Lubin S, et al. Treatment of very early rheumatoid arthritis with symptomatic therapy, disease-modifying antirheumatic drugs, or biologic agents: a cost-effectiveness analysis. *Ann Intern Med*. 2009;151: 612–21.
8. Rossi D, Modena V, Sciascia S, Roccatello D. Rheumatoid arthritis: biological therapy other than anti-TNF. *Int Immunopharmacol*. 2015;27:185–8.
9. Gupta SC, Patchva S, Aggarwal BB. Therapeutic roles of curcumin: lessons learned from clinical trials. *AAPS J*. 2013;15: 195–218.
10. Rosenbaum CC, O'Mathuna DP, Chavez M, Shields K. Antioxidants and antiinflammatory dietary supplements for osteoarthritis and rheumatoid arthritis. *Altern Ther Health Med*. 2010;16:32–40.
11. Reed GW, Leung K, Rossetti RG, Vanbuskirk S, Sharp JT, Zurier RB. Treatment of rheumatoid arthritis with marine and botanical oils: an 18-month, randomized, and double-blind trial. *Evid Based Complement Alternat Med*. 2014;2014:857456.
12. Al-Nahain A, Jahan R, Rahmatullah M. Zingiber officinale: a potential plant against rheumatoid arthritis. *Arthritis*. 2014;2014: 159089.
13. Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med*. 1977;146:857–68.
14. Bendele A, McComb J, Gould T, McAbee T, Sennello G, Chlipala E, et al. Animal models of arthritis: relevance to human disease. *Toxicol Pathol*. 1999;27:134–42.
15. Bendele A. Animal models of rheumatoid arthritis. *J Musculoskelet Neuronal Interact*. 2001;1:377–85.
16. Bendele A, McAbee T, Sennello G, Frazier J, Chlipala E, McCabe D. Efficacy of sustained blood levels of interleukin-1 receptor antagonist in animal models of arthritis: comparison of efficacy in animal models with human clinical data. *Arthritis Rheum*. 1999;42:498–506.
17. Ruff KJ, DeVore DP, Leu MD, Robinson MA. Eggshell membrane: a possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin Interv Aging*. 2009;4:235–40.
18. Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW. Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin Rheumatol*. 2009;28:907–14.
19. Danes U, Seybold M, Rittinghausen R, Triebel W, Bitterlich N. NEM® brand eggshell membrane effective in the treatment of pain associated with knee and hip osteoarthritis: results from a six center, open label German clinical study. *J Arthritis*. 2014;3:1000136–41.
20. Brunello E, Masini A. NEM® brand eggshell membrane effective in the treatment of pain and stiffness associated with osteoarthritis of the knee in an Italian study population. *Int J Clin Med*. 2016;7:169–75.
21. Wedekind KJ, Coverdale JA, Hampton TR, Atwell CA, Sorbet RH, Lunnemann J, et al. Efficacy of an equine joint supplement, and the synergistic effect of its active ingredients (chelated trace minerals and natural eggshell membrane), as demonstrated in equine, swine, and an osteoarthritis rat model. *Open Access Anim Physiol*. 2015;7:1–15.
22. Dierenfeld ES, Baum D, Hampe L, Jensen J, Atwell C, Wedekind KJ. Evaluation of a nutraceutical joint supplement in camels (*Camelus species*). *Am Holis Veterin Med Assoc J*. 2014;36:59–66.
23. Bauer KL, Dierenfeld ES, Hartup BK. Evaluation of a nutraceutical joint supplement in cranes. *Proc N Am Crane Workshop*. 2014;12:27–32.
24. Ruff KJ, DeVore DP. Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Mod Res Inflamm*. 2014;3:19–25.
25. Sim BY, Bak JW, Lee HJ, Jun JA, Choi HJ, Kwon CJ, et al. Effects of natural eggshell membrane (NEM) on monosodium iodoacetate-induced arthritis in rats. *J Nutr Health*. 2015;48:310.
26. Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the care and use of laboratory animals*. 8th ed. Washington (DC): National Academies Press; 2011.
27. Cannella AC, O'Dell JR. Traditional DMARDs: methotrexate, leflunomide, sulfasalazine, hydroxychloroquine, and combination therapies. In: Firestein GS, Budd RC, Gabriel SE, McInnes IB, O'Dell JR, eds. *Kelley and Firestein's textbook of rheumatology*. 10th ed. Philadelphia: Elsevier; 2016:958–82.e7.
28. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J*. 2008;22:659–61.
29. Ruff KJ, Endres JR, Clewell AE, Szabo JR, Schauss AG. Safety evaluation of a natural eggshell membrane-derived product. *Food Chem Toxicol*. 2012;50:604–11.
30. Landewe RB, Geusens P, van der Heijde DM, Boers M, van der Linden SJ, Garnero P. Arthritis instantaneously causes collagen type I and type II degradation in patients with early rheumatoid arthritis: a longitudinal analysis. *Ann Rheum Dis*. 2006;65:40–4.
31. Young-Min S, Cawston T, Marshall N, Coady D, Christgau S, Saxne T, et al. Biomarkers predict radiographic progression in early rheumatoid arthritis and perform well compared with traditional markers. *Arthritis Rheum*. 2007;56:3236–47.
32. Landewe RB, Geusens P, Boers M, van der Heijde D, Lems W, te KJ, et al. Markers for type II collagen breakdown predict the effect of disease-modifying treatment on long-term radiographic progression in patients with rheumatoid arthritis. *Arthritis Rheum*. 2004;50:1390–9.
33. Rousseau JC, Delmas PD. Biological markers in osteoarthritis. *Nat Clin Pract Rheumatol*. 2007;3:346–56.
34. Valdes AM, Meulenbelt I, Chassaing E, Arden NK, Bierma-Zeinstra S, Hart D, et al. Large scale meta-analysis of urinary C-terminal telopeptide, serum cartilage oligomeric protein and matrix metalloproteinase degraded type II collagen and their role in prevalence, incidence and progression of osteoarthritis. *Osteoarthritis Cartil*. 2014;22:683–9.
35. Saxne T, Heinegard D. Cartilage oligomeric matrix protein: a novel marker of cartilage turnover detectable in synovial fluid and blood. *Br J Rheumatol*. 1992;31:583–91.
36. Morozzi G, Fabbro M, Bellisai F, Pucci G, Galeazzi M. Cartilage oligomeric matrix protein level in rheumatic diseases: potential use as a marker for measuring articular cartilage damage and/or the therapeutic efficacy of treatments. *Ann N Y Acad Sci*. 2007;1108:398–407.
37. Tseng S, Reddi AH, Di Cesare PE. Cartilage oligomeric matrix protein (COMP): a biomarker of arthritis. *Biomark Insights*. 2009;4:33–44.
38. Andersson ML, Svensson B, Petersson IF, Hafstrom I, Albertsson K, Forslind K, et al. Early increase in serum-COMP is associated with joint damage progression over the first five years in patients with rheumatoid arthritis. *BMC Musculoskelet Disord*. 2013;14:229.
39. Skoumal M, Kolarz G, Klingler A. Serum levels of cartilage oligomeric matrix protein. A predicting factor and a valuable parameter for disease management in rheumatoid arthritis. *Scand J Rheumatol*. 2003;32:156–61.
40. Crnkic M, Mansson B, Larsson L, Geborek P, Heinegard D, Saxne T. Serum cartilage oligomeric matrix protein (COMP)

- decreases in rheumatoid arthritis patients treated with infliximab or etanercept. *Arthritis Res Ther.* 2003;5:R181–5.
41. Kawashiri SY, Kawakami A, Ueki Y, Imazato T, Iwamoto N, Fujikawa K, et al. Decrement of serum cartilage oligomeric matrix protein (COMP) in rheumatoid arthritis (RA) patients achieving remission after 6 months of etanercept treatment: comparison with CRP, IgM-RF, MMP-3 and anti-CCP Ab. *Joint Bone Spine.* 2010;77:418–20.
 42. Yamamoto M, Takahashi H, Ohara M, Suzuki C, Naishiro Y, Yamamoto H, et al. Evidence of cartilaginous benefit of treatment with infliximab in rheumatoid arthritis using measurement of serum COMP. *Nihon Rinsho Meneki Gakkai Kaishi.* 2007;30:41–7.
 43. Skoumal M, Haberhauer G, Feyertag J, Kittl EM, Bauer K, Dunky A. Serum levels of cartilage oligomeric matrix protein (COMP): a rapid decrease in patients with active rheumatoid arthritis undergoing intravenous steroid treatment. *Rheumatol Int.* 2006;26:1001–4.
 44. Rehman AA, Ahsan H, Khan FH. α -2-Macroglobulin: a physiological guardian. *J Cell Physiol.* 2013;228:1665–75.
 45. Tchertverikov I, Verzijl N, Huizinga TW, TeKoppele JM, Hanemaaijer R, DeGroot J. Active MMPs captured by alpha 2 macroglobulin as a marker of disease activity in rheumatoid arthritis. *Clin Exp Rheumatol.* 2003;21:711–8.
 46. Wu SM, Pizzo SV. alpha(2)-Macroglobulin from rheumatoid arthritis synovial fluid: functional analysis defines a role for oxidation in inflammation. *Arch Biochem Biophys.* 2001;391:119–26.
 47. Zapico I, Coto E, Rodriguez A, Alvarez C, Torre JC, Alvarez V. A DNA polymorphism at the alpha2-macroglobulin gene is associated with the severity of rheumatoid arthritis. *J Rheumatol.* 2000;27:2308–11.
 48. Cavalli G, Dinarello CA. Treating rheumatological diseases and co-morbidities with interleukin-1 blocking therapies. *Rheumatology (Oxford).* 2015;54:2134–44.
 49. Mertens M, Singh JA. Anakinra for rheumatoid arthritis: a systematic review. *J Rheumatol.* 2009;36:1118–25.
 50. Vincent TL, Williams RO, Maciewicz R, Silman A, Garside P. Mapping pathogenesis of arthritis through small animal models. *Rheumatology (Oxford).* 2012;51:1931–41.
 51. Haleagrahara N, Swaminathan M, Chakravarthi S, Radhakrishnan A. Therapeutic efficacy of vitamin E δ -tocotrienol in collagen-induced rat model of arthritis. *Biomed Res Int.* 2014;2014:539540.
 52. Amdekar S, Singh V, Singh R, Sharma P, Keshav P, Kumar A. *Lactobacillus casei* reduces the inflammatory joint damage associated with collagen-induced arthritis (CIA) by reducing the pro-inflammatory cytokines: *Lactobacillus casei*: COX-2 inhibitor. *J Clin Immunol.* 2011;31:147–54.
 53. Beren J, Hill SL, Diener-West M, Rose NR. Effect of pre-loading oral glucosamine HCl/chondroitin sulfate/manganese ascorbate combination on experimental arthritis in rats. *Exp Biol Med (Maywood).* 2001;226:144–51.
 54. Zhang Y, Xu W, Li H, Zhang X, Xia Y, Chu K, et al. Therapeutic effects of total alkaloids of *Tripterygium wilfordii* Hook f. on collagen-induced arthritis in rats. *J Ethnopharmacol.* 2013;145:699–705.
 55. Lv QW, Zhang W, Shi Q, Zheng WJ, Li X, Chen H, et al. Comparison of *Tripterygium wilfordii* Hook F with methotrexate in the treatment of active rheumatoid arthritis (TRIFRA): a randomised, controlled clinical trial. *Ann Rheum Dis.* 2015;74:1078–86.
 56. Benson KF, Ruff KJ, Jensen GS. Effects of natural eggshell membrane (NEM) on cytokine production in cultures of peripheral blood mononuclear cells: increased suppression of tumor necrosis factor-alpha levels after in vitro digestion. *J Med Food.* 2012;15(4):360–8.
 57. Ruff KJ, Durham PL, O'Reilly A, Long FD. Eggshell membrane hydrolyzates activate NF- κ B in vitro: possible implications for in vivo efficacy. *J Inflamm Res.* 2015;8:49–57.
 58. Stancikova M, Stancik R, Gubzova Z, Rovensky J. Collagen in the treatment of rheumatic diseases – oral tolerance. *Bratisl Lek Listy.* 1999;100:567–71.
 59. Min SY, Park KS, Cho ML, Kang JW, Cho YG, Hwang SY, et al. Antigen-induced, tolerogenic CD11c+, CD11b+ dendritic cells are abundant in Peyer's patches during the induction of oral tolerance to type II collagen and suppress experimental collagen-induced arthritis. *Arthritis Rheum.* 2006;54:887–98.
 60. Park KS, Park MJ, Cho ML, Kwok SK, Ju JH, Ko HJ, et al. Type II collagen oral tolerance; mechanism and role in collagen-induced arthritis and rheumatoid arthritis. *Mod Rheumatol.* 2009;19:581–9.
 61. Atomi Y, Shimizu M, Hasebe Y, inventors. Insulin resistance-improving agent containing eggshell membrane component, and composition using the same. US Patent 20150150916 A1. United States Patent Office. Washington, DC, USA. 2015.
 62. Williams RO. Collagen-induced arthritis in mice: a major role for tumor necrosis factor-alpha. *Methods Mol Biol.* 2007;361:265–84.
 63. Visser K, van der Heijde DM. Risk and management of liver toxicity during methotrexate treatment in rheumatoid and psoriatic arthritis: a systematic review of the literature. *Clin Exp Rheumatol.* 2009;27:1017–25.
 64. Barrera P, Laan RF, van Riel PL, Dekhuijzen PN, Boerbooms AM, van de Putte LB. Methotrexate-related pulmonary complications in rheumatoid arthritis. *Ann Rheum Dis.* 1994;53:434–9.
 65. Genestier L, Paillot R, Fournel S, Ferraro C, Miossec P, Revillard JP. Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. *J Clin Invest.* 1998;102:322–8.
 66. Seideman P, Muller-Suur R. Renal effects of aspirin and low dose methotrexate in rheumatoid arthritis. *Ann Rheum Dis.* 1993;52:613–15.
 67. Alarcon GS, Tracy IC, Strand GM, Singh K, Macaluso M. Survival and drug discontinuation analyses in a large cohort of methotrexate treated rheumatoid arthritis patients. *Ann Rheum Dis.* 1995;54:708–12.
 68. Yamanaka H. TNF as a target of inflammation in rheumatoid arthritis. *Endocr Metab Immune Disord Drug Targets.* 2015;15:129–34.
 69. Taylor PC, Feldmann M. Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis. *Nat Rev Rheumatol.* 2009;5:578–82.
 70. Houseman M, Potter C, Marshall N, Lakey R, Cawston T, Griffiths I, et al. Baseline serum MMP-3 levels in patients with rheumatoid arthritis are still independently predictive of radiographic progression in a longitudinal observational cohort at 8 years follow up. *Arthritis Res Ther.* 2012;14:R30.
 71. Uemura Y, Hayashi H, Takahashi T, Saitho T, Umeda R, Ichise Y, et al. MMP-3 as a biomarker of disease activity of rheumatoid arthritis. *Rinsho Byori.* 2015;63:1357–64.
 72. Yoshioka Y, Takahashi N, Kaneko A, Hirano Y, Kanayama Y, Kanda H, et al. Disease activity early in treatment as a predictor of future low disease activity in RA patients treated with iguratimod. *Mod Rheumatol.* 2016;26:169–74.
 73. Okamura K, Yonemoto Y, Okura C, Kobayashi T, Takagishi K. Efficacy of the clinical use of iguratimod therapy in patients with rheumatoid arthritis. *Mod Rheumatol.* 2015;25:235–40.
 74. Okamura K, Yonemoto Y, Suto T, Okura C, Takagishi K. Efficacy at 52 weeks of daily clinical use of iguratimod in patients with rheumatoid arthritis. *Mod Rheumatol.* 2015;25:534–9.
 75. Tanaka K, Yamaguchi T, Hara M. Iguratimod for the treatment of rheumatoid arthritis in Japan. *Expert Rev Clin Immunol.* 2015;11:565–73.

Effectiveness of NEM[®] brand eggshell membrane in the treatment of suboptimal joint function in dogs: a multicenter, randomized, double-blind, placebo-controlled study

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Introduction: Sub-optimal joint function is extremely prevalent in dogs. Therefore, a 6-week, prospective, randomized, double-blind, placebo-controlled study was conducted at eight different veterinary clinics to evaluate the efficacy, safety, and tolerability of NEM[®] brand eggshell membrane (EM), a novel dietary supplement shown in other species to help maintain healthy joints and connective tissues.

Subjects and methods: Fifty-one dogs received oral EM ~13.5 mg/kg (6 mg/lb) or placebo (excipients) once daily for 6 weeks. The primary outcome measure of this study was to evaluate the change in mean joint function following 1 week and 6 weeks of supplementation as determined via the Canine Brief Pain Inventory (CBPI) questionnaire (Q#5-10) in the treatment group versus the placebo group. Secondary outcome measures were for changes in mean CBPI pain and CBPI quality of life, and mean joint pain, mobility and lameness via Veterinary Canine Scoring Assessments (VCSA). A final secondary outcome measure was for a change in serum levels of the cartilage degradation biomarker, c-terminal cross-linked telopeptide of type-II collagen (CTX-II).

Results: Supplementation with EM produced a significant treatment response versus placebo at 1 week (20.5% improvement, $P=0.028$), but fell shy of significance at 6 weeks post-treatment (22.5% improvement) for the primary outcome measure (CBPI Function), despite a sizeable treatment effect. Similarly, there was also a significant treatment response versus placebo at 1 week for CBPI Pain (19.4% improvement, $P=0.010$), but fell just shy of significance at 6 weeks (22.5% improvement), again despite a sizeable treatment effect. Results were not significant versus placebo at 1 week for CBPI quality of life (14.0% improvement), but produced a significant treatment response by the end of the 6-week study (26.8% improvement, $P=0.033$). Additionally, EM produced a significant treatment response versus placebo at 6 weeks for VCSA pain (23.6% improvement, $P=0.012$), but fell shy of significance for VCSA mobility and VCSA lameness (walking & trotting). Serum CTX-II levels in EM-supplemented dogs was significantly improved versus placebo at 6 weeks (47.9% improvement, $P=0.018$). There were no serious adverse events reported during the study and subject dog owners reported that EM was well tolerated by their pets.

Conclusion: Supplementation with EM, ~13.5 mg/kg (6 mg/lb) taken once daily, significantly reduced joint pain and improved joint function rapidly (CBPI 1 week) and demonstrated a lasting improvement in joint pain (VCSA 6 weeks) leading to an improved quality of life (CBPI 6 weeks). Moreover, a profound chondroprotective effect was demonstrated following 6 weeks of supplementation with EM (CTX-II).

Keywords: EM, canine, pain, stiffness, lameness, CTX-II

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Introduction

Suboptimal joint function (stiffness, inflexibility, and lameness) is extremely prevalent in dogs, often arising from either congenital abnormalities or traumatic injury. Hip dysplasia is the most common orthopedic condition, leading to suboptimal joint function. Estimates of its rate of incidence are quite variable throughout the world, with larger studies centering around ~20% across all breeds and having a range of anywhere from a few percent to ~50% depending upon breed.¹⁻⁴ Joint laxity, femoral head misalignment, and uneven weight distribution while walking produce chronic localized inflammation in hip dysplasia, frequently leading to the development of osteoarthritis (OA) secondarily. Epidemiological evidence of the prevalence of primary and/or secondary OA in dogs is sparse, but some estimate that up to 20% of adult dogs and 80% of geriatric dogs (>8 years old) suffer from OA,^{5,6} which is in line with estimates of the prevalence of OA in humans (21%).⁷

Cartilage is primarily composed of extracellular matrix, a composite network of proteins such as type II collagen interacting with negatively charged polysaccharides such as hyaluronic acid and chondroitin sulfate (CS), all of which are synthesized and secreted by chondrocytes. Pathological conditions such as OA are characterized by an imbalance in cartilage turnover, in which catabolic processes predominate over anabolic processes. Extracellular matrix synthesis cannot keep pace with degradation, resulting in a loss of the structural integrity of the articular cartilage. This cartilage metabolism imbalance coupled with biomechanical stress in the joint resulting from laxity, misalignment, or other malformations leads to chronic inflammation and ultimately irreversible joint destruction. Products of this cartilage degradation process can be found in both blood and urine of arthritic subjects.

Of these cartilage degradation biomarkers, c-terminal cross-linked telopeptide of type II collagen (CTX-II) has been shown to have a strong correlation with the histological severity of destructive joint diseases in animal models.⁸ Because CTX-II is highly conserved genetically within vertebrate species, it has shown an exceptional correlation between animal model research and human clinical evaluation. CTX-II has been associated with both the incidence and progression of human OA in multiple clinical trials.⁹⁻¹¹ However, relatively few studies have assessed the utility of CTX-II in the evaluation of canine joint disease;¹² and to our knowledge, CTX-II has never been used to evaluate the chondroprotective effect of a treatment for naturally occurring canine joint disease.

The pain and inflammation associated with canine maladies such as hip dysplasia and OA can be quite debilitating, and few treatment options exist outside of easing symptoms. Traditional nonsurgical treatments for these disorders usually involve the use of nonsteroidal anti-inflammatory drugs (NSAIDs, eg, carprofen, meloxicam, etc).¹³ However, as in humans, there are safety concerns with long-term use of NSAIDs. Complementary and alternative medicines such as dietary supplements are also sometimes used in the management of canine OA,¹⁴ although there have been few well-controlled trials demonstrating their efficacy in dogs. Glucosamine (GluN), chondroitin, and omega-3 fatty acids (eg, fish oil, green-lipped mussel, etc) alone and in combination are widely marketed as canine dietary supplements to treat joint pain. The discovery of eggshell membrane (ESM) as a natural source of immune-modulating components has prompted the evaluation of this material as a treatment for suboptimal joint function in dogs.

ESM, found between the calcified shell and the albumin in chicken eggs, is primarily composed of fibrous proteins such as collagen type I,¹⁵ which form the mesh-like structure of the bilayered material. However, ESMs have also been shown to contain other bioactive components, namely glycosaminoglycans (ie, dermatan sulfate,¹⁶ CS,¹⁶ hyaluronic acid,¹⁷ etc). ESM is known to reduce the expression of various proinflammatory cytokines both *in vitro*¹⁸ and *in vivo*,¹⁹ including the key mediators of inflammation, interleukin-1 β and tumor necrosis factor- α . A proprietary form of ESM, commercially available as the branded product NEM[®] brand eggshell membrane (EM) (ESM Technologies LLC, Carthage, MO, USA), has demonstrated safety and efficacy in multiple clinical trials in relieving joint pain and stiffness in humans with OA²⁰⁻²² and has been investigated for similar uses in various species of animals, including cranes,²³ camels,²⁴ and horses.²⁵ However, EM has not been previously evaluated in dogs.

Although it is generally agreed that animals lack a significant placebo effect, it can nonetheless be difficult to evaluate subjective measures of their health and well-being. The Canine Brief Pain Inventory (CBPI) is a validated owner-administered test instrument (questionnaire) that is designed to assess the severity of chronic pain in dogs with OA and its impact on their function during daily activities. The CBPI has been shown to be appropriate and sufficiently sensitive to reliably detect treatment responses in multiple studies.²⁶⁻²⁹ In addition to the dog owner's assessment of treatment effect, it is also important to obtain a clinician's assessment, as a veterinarian will generally be more objective regarding the

dog's condition than will the dog owner. Over the past decade, various ordinal-scale clinician assessment tools have been developed, which provide a good basis for the inclusion of veterinarian evaluation of study dogs.^{30–32}

The multicenter trial reported herein was designed to evaluate the efficacy and safety of the natural joint treatment, EM in dogs. Therefore, a 6-week, multicenter, randomized, controlled trial was conducted to evaluate the efficacy and tolerability of EM for the treatment of suboptimal joint function (eg, stiffness, inflexibility, lameness) in dogs.

Subjects and methods

Study design

The study was conducted according to a prospective, randomized, double-blind, placebo-controlled design and was conducted across eight veterinary clinics in the Saint Louis, MO metropolitan area. The study design was approved by the Institutional Animal Care and Use Committee (IACUC) of Missouri State University (Springfield, MO USA) (Study #14-035.0) in accordance with the Guide for the Care and Use of Laboratory Animals (National Academies Press, Washington, D.C. USA, 1996). Dog owners provided their written informed consent for their dogs to participate. Eligible subjects were centrally randomized to receive either EM or placebo in the order in which they were enrolled in the study using a permuted-block randomization table consisting of four subjects per block. Dog owners, clinical investigators, subinvestigators, clinical site personnel, and the clinical coordinator (performed central randomization) were all blinded to treatment. Treatment consisted of tablets (once daily, orally) providing either 150 mg of EM or 150 mg of additional excipients for every 25 pounds of a subject dog's body weight (equating to ~6 mg/lb or 13.5 mg/kg). This dose was based upon an allometric conversion³³ from the human dose of 500 mg/d that has been shown to be effective in previous clinical trials.^{20–22} Both treatment and placebo tablets were provided by United Pet Group, a division of Spectrum Brands (Earth City, MO, USA), and were identical in appearance and odor and were stored in closed containers at ambient temperature. Inactive excipients (ie, brewer's yeast, maltodextrin, silicon dioxide) were used to produce both treatment tablets, and additional excipients replaced the EM in the placebo tablets. The placebo tablets were tested to verify that they did not contain EM. Clinic visits were scheduled for subject dogs at study initiation and at 6 weeks following the onset of treatment. Subject dog owners were given an owner-assessment diary to be filled out daily for 6 weeks and were instructed to record any changes in the overall subject health, changes in exercise routine, and any

apparent discomfort associated with ingestion of either treatment or placebo tablets. Treatment compliance was checked at the final clinic visit by owner interview and by counting the number of unused doses of the study medications.

Subjects

All privately owned dogs, 18 months of age or older, weighing 10–100 pounds whose owners were seeking relief of mild to moderate suboptimal joint function were considered for enrollment in the study. In order to be eligible, subject dogs must have had mild-to-moderate persistent suboptimal joint function (eg, difficulty in getting up from a laying position, a noticeable limp, impaired gait, difficulty in climbing stairs) lasting for at least 3 months with a mean baseline function score between 2.0 and 8.5 on Q#5–10 of the CBPI questionnaire. Dogs that were currently receiving prescription or over-the-counter analgesic medications or NSAIDs daily were only eligible to participate in the study following a 14-day washout period for NSAIDs, a 7-day washout period for narcotics, and a 90-day washout period for injected steroids. Dogs currently receiving joint health supplements or consuming a joint health diet (ie, those containing glucosamine, CS, methylsulfonylmethane, curcumin, etc) were only eligible to participate in the study following a 3-month washout period. Subjects were excluded if they were currently receiving remission-inducing drugs such as methotrexate or immunosuppressive medications or had received them within the past 3 months. They were also excluded if they had a known confounding immune-mediated (eg, lupus), known infectious (eg, Lyme disease), known neurological, or known neoplastic disease or condition that would interfere with assessment of joint function. Other exclusionary criteria were a known allergy to eggs or egg products, a significant injury to the affected joint within the past 3 months, or pregnant or nursing female dogs. Subjects participating in any other research study involving an investigational product (drug, device, or biologic) or a new application of an approved product, within 30 days of screening, were also excluded from participating in the trial.

Treatment response

The primary outcome measure of this study was to evaluate the change in mean joint function as determined via CBPI questionnaire (Q#5–10) in the treatment group versus the placebo group. An additional outcome measure was to evaluate a change in mean joint pain or discomfort as determined via CBPI questionnaire (Q#1–4) in the treatment group versus the placebo group. The treatment response end points were at 1 week (by in-home owner survey) and at the week 6 clinic

visit utilizing the eleven-question-validated CBPI questionnaire. Each of the first ten questions on the CBPI questionnaire includes a zero to ten ordinal scale, with zero equating to no pain (or does not interfere) and ten equating to extreme pain (or completely interferes). The final CBPI question asks the owner to rate the dog's overall quality of life (QOL) using a five-category Likert-type scale (poor/fair/good/very good/excellent), which was then converted to a numeric value (1–5) for statistical comparison. End points were then compared to placebo assessments. Additional outcome measures were the change in mean joint pain and mobility utilizing a Veterinary Canine Scoring Assessment (VCSA) and the change in mean lameness while walking (w) and trotting (t) utilizing a second VCSA. The joint pain and mobility VCSA consisted of a five-point ordinal scale assessment via palpation or manipulation of the most apparent affected joint or joints (Table 1), and the lameness VCSA consisted of a seven-point ordinal scale assessment while either walking or trotting (Table 2).

Assessment of serum CTX-II

A secondary objective of this study was to evaluate the change in mean serum CTX-II levels in the treatment group versus placebo at 6 weeks. Blood samples were collected in serum tubes at baseline and at the week 6 clinic visit. In brief, the blood samples were allowed to clot at room temperature for 15–30 minutes, followed by centrifugation at 1,000–2,000×g for ~10 minutes. Following transfer of the supernatant serum to a new tube, samples were stored frozen (–20°C) until analysis. Serum concentrations of CTX-II were measured via enzyme-linked immunosorbent assay using a commercial immunoassay (Serum Pre-Clinical CartiLaps® [CTX-II] EIA; Immunodiagnostic Systems, Inc., Gaithersburg, MD, USA) according to manufacturer's instructions using a SpectraMax Plus 384 microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). Samples were run in duplicate when assayed.

Adverse events and safety

A final objective of this study was to evaluate safety, tolerability, and any adverse reactions associated with supplementation with EM. Blood samples were collected at baseline and at the week 6 clinic visit to evaluate treatment safety via clinical chemistry (total protein, albumin, globulin, albumin/globulin ratio, blood urea nitrogen, creatinine, blood urea nitrogen/creatinine ratio, glucose, alanine aminotransferase, and alkaline phosphatase) and hematology (platelet count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin count, red blood cell count, and white blood cell count with differential

Table 1 Veterinary Canine Scoring Assessment (VCSA) for joint pain and mobility

Criterion	Clinical scoring
Pain on palpation	1. None 2. Mild signs; dog turns head in recognition 3. Moderate signs; dog pulls limb away 4. Severe signs; dog vocalizes or becomes aggressive 5. Dog will not allow palpation
Joint mobility	1. Full range of motion 2. Mild limitation (10%–20%) in range of motion; no crepitus 3. Mild limitation (10%–20%) in range of motion; with crepitus 4. Moderate limitation (20%–50%) in range of motion; ±crepitus 5. Severe limitation (>50%) in range of motion; ±crepitus

Table 2 Veterinary Canine Scoring Assessment (VCSA) for lameness when walking and trotting

Criterion	Clinical scoring
Lameness when walking	1. No detectable lameness 2. Slight intermittent weight-bearing lameness (difficult to see) 3. Moderate intermittent weight-bearing lameness (easy to see) 4. Persistent weight-bearing lameness (obvious pain) 5. Persistent lameness, occasionally nonweight bearing 6. Persistent nonweight-bearing lameness (willing to move) 7. Severe persistent nonweight-bearing lameness (reluctant to move)
Lameness when trotting	1. No detectable lameness 2. Slight intermittent weight-bearing lameness (difficult to see) 3. Moderate intermittent weight-bearing lameness (easy to see) 4. Persistent weight-bearing lameness (obvious pain) 5. Persistent lameness, occasionally nonweight bearing 6. Persistent nonweight-bearing lameness (willing to move) 7. Severe persistent nonweight-bearing lameness (reluctant to move)

[lymphocytes, monocytes, neutrophils, eosinophils, and basophils]). Clinical chemistry testing was performed by a commercial veterinary testing laboratory (Antech Diagnostics, Chesterfield, MO, USA). The owner-assessment diaries were also reviewed, and any discomfort or other adverse events (AEs) were recorded and reported in accordance with the study protocol. AEs and serious AEs were assessed by the clinical investigator at each study visit and followed until resolution, as necessary. Serious AEs were required to be reported to the study monitor immediately.

Statistical analysis

As this is the first efficacy trial in dogs, historical data were not available to serve as a basis for sample size determination. However, the hypothesis for this study is that the treatment group will be superior to that of the placebo group in improving suboptimal joint function. A 15% absolute change in the mean primary treatment response (joint function score will decrease by an average of at least 15% in the treatment group versus the placebo group) was used for sample size determination. Based upon this, it was estimated that a sample size of 40 dogs (20 treatment and 20 placebo) would need to be enrolled to provide the study with a statistical power of 80% to detect a treatment effect difference between the treatment group and the placebo group, assuming a rate of response of 20% in the treatment group, a rate of response of 5% in the placebo group, and a withdrawal rate of 5%. Since the enrollment for the study was 51 dogs, this should be sufficient to provide adequate safety and comparative effectiveness information. Descriptive statistics were calculated, including mean age and weight, and comparisons of these demographic data from the eight clinical sites were made with a Kruskal–Wallis test for multiple independent samples at baseline to validate randomization. Within-group comparisons, using the Kruskal–Wallis test for multiple independent samples, were also made within clinical sites to rule out any site bias. Post-baseline statistical analyses were performed as repeated measures analysis of variance. The items found to have statistical significance with repeated measures analysis of variance were then compared using a Wilcoxon test for dependent samples. In all cases, statistical significance was accepted at $P < 0.05$. Analysis of the primary end point and all secondary end points was conducted on the intent-to-treat population (ie, including all randomized subjects with at least one efficacy assessment after randomization). The last observation carried forward approach was used for subjects that made at least one follow-up visit but that did not complete the study (lost to follow-up) to minimize missing data points for statistical transformations. SYSTAT software (Version 13) was used for all statistical analyses.³⁴

Results

Subject recruitment began in August 2014 at eight veterinary clinics in the Saint Louis, Missouri metropolitan area, and the final evaluation was completed in August 2015. A total of 51 dogs between the ages of 3 years and 14 years with suboptimal joint function were enrolled in the study and underwent randomization. Of these subjects, 12% (6/51) were from site 1, 20% (10/51) were from site 2, 12% (6/51) were from site 3, 25% (13/51) were from site 4, 10% (5/51)

were from site 5, 8% (4/51) were from site 6, 10% (5/51) were from site 7, 4% (2/51) were from site 8, 21 (41%) were female, 30 (59%) were male, and all were either spayed or neutered. The treatment joints consisted of stifle/knee (24), hip (21), shoulder (3), and elbow (10). Of the 24 subjects in which the stifle/knee was the affected joint, six (25%) had bilateral incidence. Of the 21 subjects in which the hip was the affected joint, eleven (52%) had bilateral incidence. Complete subject demographics, subdivided by treatment group, are reported in Table 3. Forty-six dogs completed the 6-week study per the protocol. The dropout rate (9.8%; three placebo and two treatment) was moderately higher than the estimated dropout rate (5%) used in the sample size determination; however, enrollment well exceeded the sample size of 40 dogs. From each group, two dogs were withdrawn due to lack of efficacy, and one dog was withdrawn from the placebo group due to not meeting the inclusion criteria. Compliance with the study treatment regimen was good in both treatment groups.

Subject demographic data were initially evaluated to ensure randomization within each site (not shown). Additionally, subject data were evaluated between sites to exclude site bias (not shown). During the randomization evaluation, data from site 1 exhibited some abnormalities, and upon further investigation, randomization could not be guaranteed so data from these dogs (6) were excluded from further evaluation. As there were no abnormalities within the remaining sites, the data (45) were pooled for all subsequent analyses. A clinical comparison of valid subjects (excluding noncompliance) was carried out to obtain mean baseline values for each of the CBPI outcome measures (Table 4). Statistical analysis of the

Table 3 Subject demographics

	Treatment	Placebo
Age, years	9.5±3.4	9.5±2.2
Sex		
Male (%)	17 (65)	13 (52)
Female (%)	9 (35)	12 (48)
Weight, kg (lbs)	23±11 (51±25)	25±11 (55±25)
Breeds		
Pure (%)	15 (58)	13 (52)
Mixed (%)	11 (42)	12 (48)
Affected joint		
Stifle/knee (l, r, bilateral)	7 (1, 4, 2)	17 (6, 7, 4)
Hip (l, r, bilateral)	13 (4, 3, 6)	8 (2, 1, 5)
Shoulder (l, r, bilateral)	2 (2, 0, 0)	1 (0, 1, 0)
Elbow (l, r, bilateral)	6 (3, 2, 1)	4 (2, 1, 1)

Note: Except where indicated otherwise, values are reported as mean ± standard deviation (n=51).

Abbreviations: l, left, r right.

primary outcome measure (CBPI function) revealed that supplementation with EM produced a significant treatment response versus placebo at 1 week (20.5% improvement, $P=0.028$), but fell short of significance at 6 weeks post-treatment (21.1% improvement, $P=0.155$), despite a sizeable treatment effect. Similarly, supplementation with EM produced a significant treatment response versus placebo at 1 week for CBPI pain (19.4% improvement, $P=0.010$), but fell just short of significance at 6 weeks (22.5% improvement, $P=0.098$), again despite a sizeable treatment effect. Supplementation with EM was not significant versus placebo at 1 week for CBPI QOL (14.0% improvement, $P=0.155$), but produced a significant treatment response by the end of the 6-week study (26.8% improvement, $P=0.033$). A clinical comparison of valid subjects (excluding noncompliance) was also carried out to obtain mean baseline values for each of the VCSA outcome measures (Table 5). Statistical analysis of the secondary outcome measures (VCSA pain, mobility, lameness while walking, and lameness while trotting) revealed that supplementation with EM produced a significant treatment response versus placebo at 6 weeks for VCSA pain (23.6% improvement, $P=0.012$), but fell short of significance for VCSA mobility (11.7% improvement, $P=0.141$), VCSA lameness (w, 9.4% improvement, $P=0.329$), and VCSA lameness (t, 10.8% improvement, $P=0.358$).

Table 4 Mean scores for CBPI owner evaluations and absolute treatment effect in NEM® brand eggshell membrane (EM)-supplemented and placebo groups at baseline, 1 week, and 6 weeks post-treatment

	Weeks post-treatment	Treatment		Absolute treatment effect (%)
		Placebo	EM	
CBPI pain	Baseline (n=22, 23)	4.0±1.6	4.5±2.0	N/A
	1 (n=21, 21)	3.9±1.9	3.5±2.0	-19.4*
	6 (n=21, 21)	3.9±1.6	3.3±2.2	-22.5
CBPI function	Baseline (n=22, 23)	5.1±2.1	5.2±2.2	N/A
	1 (n=21, 21)	4.7±2.4	3.7±2.3	-20.5*
	6 (n=21, 21)	4.5±2.5	3.4±2.7	-21.1
CBPI QOL	Baseline (n=22, 23)	3.0±0.6	2.7±0.9	N/A
	1 (n=21, 21)	3.0±0.7	3.1±0.7	+14.0
	6 (n=21, 21)	3.2±0.7	3.6±1.1	+26.8*

Notes: Except where indicated otherwise, values are reported as mean \pm standard deviation. Absolute treatment effect is the net difference of treatment versus placebo for the change in mean treatment effect from baseline expressed in percentage. Negative values for pain or function indicate superior improvement in the treatment group, whereas positive values for QOL indicate superior improvement in the treatment group. P -values were determined by repeated measures analysis of variance and represent treatment versus placebo. * $P<0.05$.

Abbreviations: CBPI, Canine Brief Pain Inventory; N/A, not applicable; QOL, quality of life.

Table 5 Mean scores for VCSA veterinarian evaluations and absolute treatment effect in NEM® brand eggshell membrane (EM)-supplemented and placebo groups at baseline and 6 weeks post-treatment

	Weeks post-treatment	Treatment		Absolute treatment effect (%)
		Placebo	EM	
VCSA pain	Baseline (n=22, 23)	2.0±0.7	2.8±0.4	N/A
	6 (n=21, 21)	2.1±0.9	2.2±0.7	-23.6*
VCSA mobility	Baseline (n=22, 23)	2.5±0.7	2.9±1.0	N/A
	6 (n=21, 21)	2.5±0.9	2.5±1.1	-11.7
VCSA lameness (w)	Baseline (n=22, 23)	2.7±0.9	2.9±1.0	N/A
	6 (n=21, 21)	2.4±1.2	2.3±0.9	-9.4
VCSA lameness (t)	Baseline (n=22, 23)	2.9±1.0	2.7±1.0	N/A
	6 (n=21, 21)	2.7±1.3	2.3±1.0	-10.8

Notes: Except where indicated otherwise, values are reported as mean \pm standard deviation. Absolute treatment effect is the net difference of treatment versus placebo for the change in mean treatment effect from baseline expressed in percentage. Negative values indicate superior improvement in the treatment group. P -values were determined by repeated measures analysis of variance and represent treatment versus placebo. * $P<0.05$.

Abbreviations: VCSA, Veterinary Canine Scoring Assessment; N/A, not applicable; w, walking; t, trotting.

Viable serum samples from both baseline and week 6 were obtained from 26 (14 treatment and 12 placebo) of the 42 dogs that completed the study. A clinical comparison of these valid subjects was carried out to obtain mean baseline values for the cartilage degradation biomarker, CTX-II. Statistical analysis of serum CTX-II levels revealed that supplementation with EM produced a significant treatment response versus placebo at 6 weeks (47.9% improvement, $P=0.018$; Figure 1; placebo, baseline: 5.0±6.9 pg/mL and week 6: 6.9±7.5 pg/mL; EM, baseline: 5.0±11.6 pg/mL and week 6: 4.5±9.0 pg/mL). The intra-assay coefficient of variation was 5.13.

There were 15 AEs reported during the study. These were composed of three instances of loose stool, two instances of diarrhea, seven instances of vomiting, two instances of rash, and one instance of constipation. Eight AEs occurred in five placebo group subjects and seven AEs occurred in four treatment group subjects. All of the AEs were deemed unrelated to the study treatment. There were no serious AEs reported during the study. There were no treatment-related abnormalities in any of the clinical chemistry parameters evaluated in the study. Subject dog owners reported that the treatment was well tolerated by their pets.

Discussion

Joint and connective tissue disorders are extremely common in dogs and can have a significant impact on the QOL

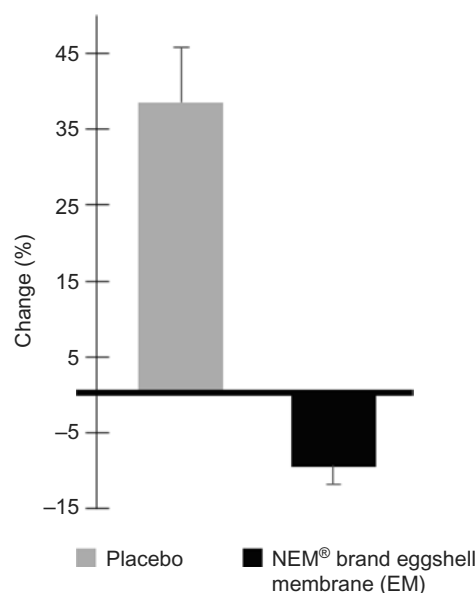


Figure 1 Percentage of change from baseline in serum CTX-II levels after 6 weeks in EM-supplemented and placebo groups.

Abbreviation: CTX-II, cross-linked telopeptide of type II collagen.

for those that suffer from the debilitating conditions. This trial was designed to evaluate the efficacy, safety, and tolerability of EM ESM as a natural treatment alternative for dogs suffering from suboptimal joint function. Results of this study demonstrate that EM is both effective and safe for treating pain (CBPI and VCSA) and immobility (CBPI) associated with suboptimal joint function and effectively improves the QOL (CBPI) of the dogs receiving the supplement.

Subject dogs experienced a relatively rapid (1 week) response as rated by CBPI pain with a mean improvement of 19.4%. By the end of the 6-week follow-up period, the mean response for joint pain improved to 23.6% as judged by veterinarians (VCSA). By comparison, Moreau et al³⁰ found no benefit in dogs with OA even after 8 weeks of treatment with a supplement containing glucosamine (GluN) hydrochloride and CS. McCarthy et al³² found a similar treatment response from a Glu/CS supplement to that found from EM presented here; however, this benefit was only seen after >9 weeks of supplementation. Importantly, both studies utilized veterinarian assessments nearly identical to our VCSA. The rapid onset of the treatment effect from EM is on par with that found for meloxicam and carprofen evaluated in these same studies. A brief responder analysis of the pain-related data from the current study provides a number of clinically relevant highlights. Approximately one-half (48%) of the EM-treated subjects experienced a $\geq 33\%$ improvement in both VCSA pain and CBPI pain by 6 weeks (not shown). Importantly, the owner assessments of

pain were corroborated by the veterinarian assessments of pain, and these results align well with results from previous responder analyses in clinical studies of EM that were conducted in humans.^{20–22}

Subject dogs also experienced a relatively rapid (1 week) response as rated by CBPI function with a mean improvement of 20.5%. By the end of the 6-week follow-up period, the mean response for CBPI function improved to 21.1%. The sizeable improvements noted by the dog owners could not be fully corroborated by the veterinarian assessments of mobility and lameness (w and t), which improved by an average of 11.7%, 9.4%, and 10.8%, respectively. This disagreement may be a consequence of the difference in precision between the two instruments. That is, the CBPI questionnaire utilizes a ten-point scale, whereas the VCSA questionnaires utilize a five-point scale (mobility) or seven-point scale (lameness). Therefore, a more substantial change in joint function is required to result in a change in VCSA scoring. It is also possible that the disagreement in instruments arises from the inherent design of the questionnaires. That is, the VCSA questionnaires are more specific in their assessment of joint function, whereas the CBPI questionnaire evaluates joint function more broadly and generally.

The effect a treatment has on overall QOL can be an important determinant of treatment efficacy and ultimately future treatment compliance. Although joint pain, immobility, and lameness certainly factor into QOL, there are also certain intangible qualities that affect this aspect of treatment, as well. Importantly, in the present study, dog owners reported a rapid (1 week) response in CBPI QOL with a mean improvement of 14.0%. By the end of the 6-week follow-up period, the mean response for CBPI QOL improved to 26.8%. The magnitude of this improvement would be expected to be clinically meaningful in the QOL of dogs suffering from suboptimal joint function.

Symptom relief is certainly a critical component of any arthritis treatment. However, the further capacity to reduce inflammation within the joint and preserve articular cartilage integrity – to be disease modifying – is lacking in currently available treatments. We report here for the first time the chondroprotective effects in dogs, as evidenced by the substantial reduction (47.9%) in the change in serum CTX-II levels after 6 weeks of supplementation with EM versus placebo. The capacity of EM to impact CTX-II so profoundly was first shown in a rat model of OA;³⁵ however, the current study is the first evidence demonstrated in naturally occurring joint disease. Evidence from prior studies

involving EM indicates that this chondroprotective effect likely arises from reduced joint inflammation^{18,19} coupled with reduced levels of various cartilage-degrading matrix metalloproteinases.³⁵

The safety profile for EM is also of significance as there have been no reports of serious AEs associated with treatment in any of the clinical studies conducted to date. No side effects from consuming EM have thus far been identified, excluding the obvious egg allergy concern. This is of obvious importance in canine conditions such as hip dysplasia and OA that require long-term treatment.

The trial had a somewhat limited enrollment (51 subjects); however, there was a fairly low drop-out rate (9.8%) and good treatment compliance. The variability in the severity of the suboptimal joint function in the study dogs likely made it more difficult to detect treatment responses, and this was complicated by the wide variety of breeds (and concurrent sizes) of dogs enrolled in the study, as well. The owner assessment (CBPI), although validated for use in the treatment of OA with NSAIDs, appeared to effectively detect changes in joint pain and joint function in this study. The veterinarian assessments (VCSA), although based upon previous assessment designs,^{29–31} were modified to a significant degree for this study and appeared to be effective tools for clinician assessment. The addition of additional objective measures of joint function (eg, force plate analysis, gait analysis) could prove beneficial. Further research is warranted to validate the use of serum CTX-II as a diagnostic or prognostic biomarker for canine cartilage status.

With so many dogs suffering from suboptimal joint function, it is important for dog owners to have treatment options that are both safe and effective. The reporting of the results from this eight-center randomized controlled trial demonstrates that EM ESM is a viable therapeutic option for the management of the pain and loss of function associated with suboptimal joint function in dogs. Supplementation with EM, 6 mg/lb (~13.5 mg/kg) taken once daily, significantly reduced joint pain and improved joint function rapidly (CBPI 1 week) and demonstrated a lasting improvement in joint pain (VCSA 6 weeks) leading to an improved QOL (CBPI 6 weeks). Moreover, a profound chondroprotective effect was demonstrated following 6 weeks of supplementation with EM. There were also clinically meaningful results from a brief responder analysis, demonstrating that a significant proportion of treated dogs will benefit substantially from EM supplementation.

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Disclosure

KJR and MB are employed by the sponsor. KJK is a paid consultant for the sponsor. The other authors report no conflicts of interest in this work.

References

1. Coopman F, Verhoeven G, Saunders J, Duchateau L, van Bree H. Prevalence of hip dysplasia, elbow dysplasia and humeral head osteochondrosis in dog breeds in Belgium. *Vet Rec.* 2008;163(22):654–658.
2. Rettenmaier JL, Keller GG, Lattimer JC, Corley EA, Ellersjeck MR. Prevalence of hip dysplasia in a veterinary teaching hospital population. *Vet Radiol Ultrasound.* 2002;43(4):313–318.
3. Stanin D, Pavlak M, Vrbanc Z, Potočnjak D. Prevalence of hip dysplasia in dogs according to official radiographic screening in Croatia. *Veterinarski Arhiv.* 2011;81(2):235–248.
4. Simon S, Ganesh MR, Ayyappan S, et al. Incidence of canine hip dysplasia: a survey of 272 cases. *Vet World.* 2010;3(5):219–220.
5. Marshall WG, Bockstahler BA, Hulse DA, Carmichael S. A review of osteoarthritis and obesity: current understanding of the relationship and benefit of obesity treatment and prevention in the dog. *Vet Comp Orthop Traumatol.* 2009;22(5):339–345.
6. Rialland P, Bichot S, Moreau M, et al. Clinical validity of outcome pain measures in naturally occurring canine osteoarthritis. *BMC Vet Res.* 2012;8:162.
7. Helmick CG, Felson DT, Lawrence RC, et al. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part I. *Arthritis Rheum.* 2008;58(1):15–25.
8. Oestergaard S, Chouinard L, Doyle N, et al. The utility of measuring C-terminal telopeptides of collagen type II (CTX-II) in serum and synovial fluid samples for estimation of articular cartilage status in experimental models of destructive joint diseases. *Osteoarthritis Cartilage.* 2006;14(7):670–679.
9. Valdes AM, Meulenbelt I, Chassaing E, et al. Large scale meta-analysis of urinary C-terminal telopeptide, serum cartilage oligomeric protein and matrix metalloproteinase degraded type II collagen and their role in prevalence, incidence and progression of osteoarthritis. *Osteoarthritis Cartilage.* 2014;22(5):683–689.
10. van Spil WE, DeGroot J, Lems WF, Oostveen JCM, Lafeber FPJG. Serum and urinary biochemical markers for knee and hip-osteoarthritis: a systematic review applying the consensus BIPED criteria. *Osteoarthritis Cartilage.* 2010;18(5):605–612.
11. Patraa D, Sandell LJ. Recent advances in biomarkers in osteoarthritis. *Curr Opin Rheumatol.* 2011;23(5):465–470.
12. Dam EB, Byrjalsen I, Karsdal MA, Qvist P, Christiansen C. Increased urinary excretion of C-telopeptides of type II collagen (CTX-II) predicts cartilage loss over 21 months by MRI. *Osteoarthritis Cartilage.* 2009;17(3):384–389.

13. Hurlbeck C, Einspanier R, Pfeil I, Bondzio A. Evaluation of biomarkers for osteoarthritis caused by fragmented medial coronoid process in dogs. *Res Vet Sci*. 2014;96(3):429–435.
14. Henrotin Y, Sanchez C, Balligand M. Pharmaceutical and nutraceutical management of canine osteoarthritis: present and future perspectives. *Vet J*. 2005;170(1):113–123.
15. Wong M, Hendrix MJC, von der Mark K, Little C, Stern R. Collagen in the egg shell membranes of the hen. *Dev Biol*. 1984;104(1):28–36.
16. Baker JR, Balch DA. A study of the organic material of hen's-egg shell. *Biochem J*. 1962;82:352–361.
17. Long FD, Adams RG, DeVore DP, Inventors. Preparation of hyaluronic acid from eggshell membrane. 6946551. 2005 Sep 20.
18. Benson KF, Ruff KJ, Jensen GS. Effects of natural eggshell membrane (NEM) on cytokine production in cultures of peripheral blood mononuclear cells: increased suppression of tumor necrosis factor- α levels after in vitro digestion. *J Med Food*. 2012;15(4):360–368.
19. Ruff KJ, DeVore DP. Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Mod Res Inflamm*. 2014;3(1):19–25.
20. Ruff KJ, DeVore DP, Leu MD, Robinson MA. Eggshell membrane: a possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin Interv Aging*. 2009;4:235–240.
21. Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW. Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin Rheumatol*. 2009;28(8):907–914.
22. Danesch U, Seybold M, Rittinghausen R, Treibel W, Bitterlich N. NEM® brand eggshell membrane effective in the treatment of pain associated with knee and hip osteoarthritis: results from a six-center, open-label German Clinical Study. *J Arthritis*. 2014;3(3):136.
23. Bauer KL, Dierenfeld ES, Hartup BK. Evaluation of a nutraceutical joint supplement in cranes. *Proc North Am Crane Workshop*. 2014;12: 27–32.
24. Dierenfeld ES, Baum D, Hampe L, Jensen J, Atwell C, Wedekind K. Evaluation of a nutraceutical joint supplement in camels. *Am Holistic Vet Med Assoc J*. 2014;39:59–66.
25. Wedekind KJ, Coverdale JA, Hampton TR, et al. Efficacy of an equine joint supplement, and the synergistic effect of its active ingredients (chelated trace minerals and natural eggshell membrane), as demonstrated in equine, swine, and an osteoarthritis rat model. *Open Access Anim Physiol*. 2015;7:13–27.
26. Brown DC, Boston RC, Coyne JC, Farrar JT. Development and psychometric testing of an instrument designed to measure chronic pain in dogs with osteoarthritis. *Am J Vet Res*. 2007;68(6):631–637.
27. Brown DC, Boston RC, Coyne JC, Farrar JT. Ability of the Canine Brief Pain Inventory to detect response to treatment in dogs with osteoarthritis. *J Am Vet Med Assoc*. 2008;233(8):1278–1283.
28. Brown DC, Boston RC, Farrar JT. Comparison of force plate gait analysis and owner assessment of pain using the canine brief pain inventory in dogs with osteoarthritis. *J Vet Intern Med*. 2013;27(1):22–30.
29. Brown DC, Bell M, Rhodes L. Power of treatment success definitions when the Canine Brief Pain Inventory is used to evaluate carprofen treatment for the control of pain and inflammation in dogs with osteoarthritis. *Am J Vet Res*. 2013;74(12):1467–1473.
30. Moreau M, Dupuis J, Bonneau NH, Desnoyers M. Clinical evaluation of a nutraceutical, carprofen and meloxicam for the treatment of dogs with osteoarthritis. *Vet Rec*. 2003;152(11):323–329.
31. Innes JF, Fuller CJ, Grover ER, Kelly AI, Burn JF. Randomised, double-blind, placebo-controlled parallel group study of P54FP for the treatment of dogs with osteoarthritis. *Vet Rec*. 2003;152(15):457–460.
32. McCarthy G, O'Donovan J, Jones B, McAllister H, Seed M, Mooney C. Randomised double-blind, positive-controlled trial to assess the efficacy of glucosamine/chondroitin sulfate for the treatment of dogs with osteoarthritis. *Vet J*. 2007;174(1):54–61.
33. U.S. Food and Drug Administration [webpage on the Internet]. *Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. 2005. Available from: <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>. Accessed January 3, 2014.
34. Systat Software, Inc [homepage on the Internet]. Available from: www.systat.com. Accessed September 14, 2013.
35. Sim BY, Bak JW, Lee HJ, et al. Effects of natural eggshell membrane (NEM) on monosodium iodoacetate-induced arthritis in rats. *J Nutr Health*. 2015;48(4):310–318.

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NEM® Brand Eggshell Membrane Effective in the Treatment of Pain and Stiffness Associated with Osteoarthritis of the Knee in an Italian Study Population

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Abstract

A single-center, open-label clinical study was conducted to evaluate the efficacy and safety of NEM® as a natural treatment for pain and stiffness associated with osteoarthritis of the knee in an Italian population. NEM® brand eggshell membrane is a unique dietary supplement that contains naturally occurring glycosaminoglycans and proteins essential for maintaining healthy joints and connective tissues. Twenty-five subjects received oral NEM®, 500 mg once daily for four weeks. The primary outcome measure was to evaluate the mean effectiveness of NEM® in relieving general pain associated with moderate osteoarthritis of the knee at 10 and 30 days utilizing a 10-question, abbreviated questionnaire based on the WOMAC osteoarthritis questionnaire. Supplementation with NEM® produced a significant treatment response from baseline at both 10 days and 30 days for composite pain (40.6% reduction, $p < 0.001$; 66.4% reduction, $p < 0.001$, respectively). There was also a statistically significant concurrent reduction in analgesic use during the 30-day study period. Additionally, a significant treatment response from baseline was also observed for composite stiffness at both 10 days and 30 days (22.2% reduction, $p = 0.009$; 59.7% reduction, $p < 0.001$, respectively). There were no adverse events or serious adverse events reported during the study and the treatment was reported to be well tolerated by study participants. NEM® is an effective and safe natural therapeutic option for the treatment of both pain and stiffness associated with osteoarthritis of the knee. Supplementation with NEM®, 500 mg taken once daily, significantly reduced both pain and stiffness rapidly (10 days) and this effect continued to improve through 30 days. There was also a meaningful reduction in the amount of analgesic consumed on a weekly basis, which further enhanced patients' safety.

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Keywords

Knee, Osteoarthritis, Supplement, Egg Shell Membrane, Glycosaminoglycans

1. Introduction

Osteoarthritis (OA) is a degenerative disease primarily affecting the cartilage of articular joints and is frequently accompanied by varying degrees of joint pain and stiffness in afflicted subjects. OA is one of the most common causes of chronic pain in adults 65 and older and often leads to disability as the disease progresses [1]. Estimates of the prevalence of OA in European populations vary widely, however, two recent studies conducted in Italy found the prevalence of knee OA to be 29.8% [2] and 36.7% [3]. The pain associated with these maladies can be quite debilitating and few treatment options exist outside of easing symptoms. This usually involves the use of analgesics (e.g. acetaminophen, hydrocodone) or non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ibuprofen, celecoxib, etc.), alone or in combination. Most of these treatments have shown limited effectiveness in randomized controlled clinical trials (RCTs) [4]-[6] or are known to have significant and sometimes severe side effects [7] [8]. NEM[®] brand eggshell membrane has previously demonstrated good efficacy in relieving joint pain and stiffness in multiple clinical trials in the U.S. [9] [10] and recently in a German population [11].

Eggshell membrane is primarily composed of fibrous proteins such as Collagen Type I [12]. However, eggshell membranes have also been shown to contain other bioactive components, namely glycosaminoglycans (*i.e.* dermatan sulfate [13], chondroitin sulfate [13], hyaluronic acid [14], etc). ESM Technologies, LLC (Carthage, MO, USA) has developed methods to efficiently and effectively separate eggshell membrane from eggshells on a commercial metric-ton scale. The isolated membrane is then partially hydrolyzed using a proprietary process and dry-blended to produce NEM[®] brand eggshell membrane. Compositional analysis of NEM[®] conducted by ESM Technologies has identified a high content of protein and moderate quantities of glucosamine (up to 1% by dry weight), chondroitin sulfate (up to 1%), hyaluronic acid (up to 2%), and collagen (Type I, up to 5%).

The single-center trial reported here was designed to evaluate the efficacy of this natural arthritis treatment in an Italian population and to confirm the results found previously in the U.S. and Germany. Therefore, a 1-month open-label study was conducted at a single clinical site in Italy to evaluate the efficacy and tolerability of NEM[®] for the relief of the pain and discomfort associated with osteoarthritis of the knee.

2. Patients and Methods

2.1. Study Design

The study was conducted according to a prospective, single-center, open-label design and was conducted in Italy in accordance with the International Conference on Harmonization guideline for the principles of Good Clinical Practice (ICH E6) and the Declaration of Helsinki. Patients provided their written informed consent to participate. The clinical investigators were not blinded to treatment (open-label). Treatment consisted once daily orally of 500 mg of NEM[®] in vegetarian capsules that were stored in closed containers at ambient temperature. Clinic visits were scheduled for subjects at study initiation and at 10 days and 30 days following the onset of treatment. Treatment compliance was checked at clinic visits by patient interview and by counting the number of unused doses of the study medications. Analgesics (*i.e.* acetaminophen) were allowed for pain relief, as needed. Subjects recorded the time and amount of analgesic taken in patient diaries.

2.2. Patients

All subjects 18 years of age or older who were seeking relief of mild to moderate pain due to osteoarthritis of the knee were considered for enrollment in the study. In order to be eligible, subjects must have had moderate persistent pain in the knee associated with osteoarthritis and must have had baseline scores within the range of 4 - 7 on questions 1, 2, & 5 dealing with joint pain. Subjects that were currently taking analgesic medications daily, currently taking glucosamine, chondroitin sulfate, MSM, or collagen were ineligible to participate in the study. Patients were excluded if they were currently receiving remission-inducing drugs such as methotrexate or im-

munosuppressive medications or had received them within the past 3 months. Other exclusionary criteria were: a known allergy to eggs or egg products, or pregnant or breastfeeding women. Subjects participating in any other research study involving an investigational product (drug, device, or biologic) or a new application of an approved product, within 30 days of screening were also excluded from participating in the trial.

2.3. Location of Patients

The majority of patients were enrolled in an area of 50 km around the city of Verona, the capital of the homonymous province, where the Medical center REGENESIS is located, others patients come from different provinces, in particular Trento, Vicenza, Padova and Pordenone. The map below shows the different origins of the patients.

2.4. Treatment Response

The primary outcome measure of this study was to evaluate the mean effectiveness of NEM[®] in relieving general pain associated with moderate osteoarthritis of the knee (composite score of Questions 1 - 8). A composite score was calculated as the sum of the questions of interest. Additional outcome measures were to evaluate general stiffness (composite score of Questions 9 & 10), analgesic use during the study, and non-composite mean results for all 10 individual questions. The primary treatment response endpoints were the 10-and 30-day patient assessments utilizing a 10-question short-form questionnaire (see **Figure 1**) derived from the Western Ontario & McMaster Universities Osteoarthritis Index (WOMAC) questionnaire. Each question included a zero to 10 analog Likert-scale, with zero equating to no pain (or no stiffness) and 10 equating to most severe pain (or most severe stiffness). Patients were asked to mark a number corresponding to the perceived pain (or stiffness) from the affected treatment joint (s). Endpoints were then compared to pretreatment assessments.

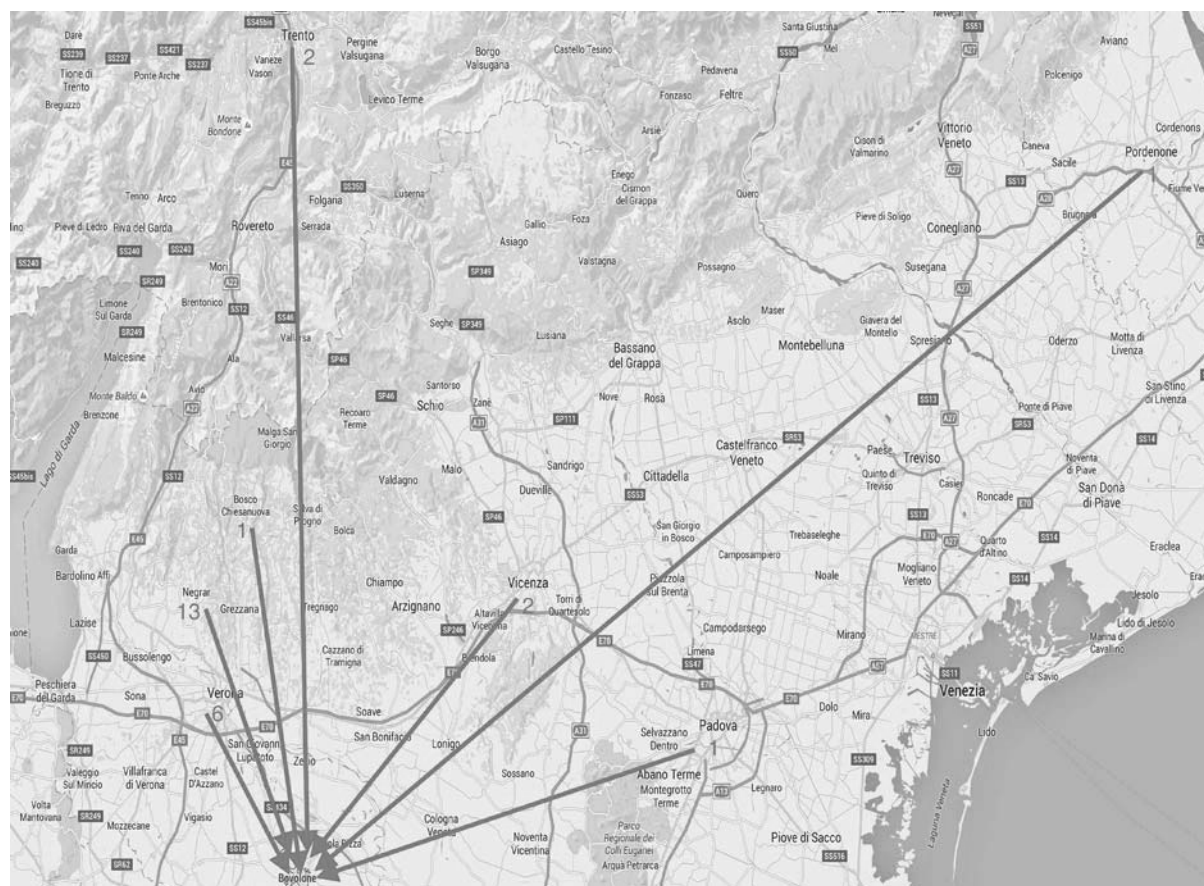


Figure 1. Questions used in the short-form questionnaire completed by study participants.

-
- Question 1: *Pain when walking on level ground?*
- Question 2: *Pain when going up or down stairs?*
- Question 3: *Pain while at rest (i.e. sitting, lying down, etc.)?*
- Question 4: *Pain when sitting with legs bent for an extended period of time (i.e. in a car, at a theater, etc.)?*
- Question 5: *Pain when getting up from a seated position?*
- Question 6: *Pain when getting in and out of a car, a bathtub, etc.?*
- Question 7: *Pain when bending, stooping, or kneeling?*
- Question 8: *Pain when putting on socks or pantyhose?*
- Question 9: *Stiffness when first getting up from bed in the morning?*
- Question 10: *Stiffness when sitting, laying, or resting later in the day?*
-

2.5. Adverse Events

A secondary objective of this study was to evaluate tolerability and any adverse reactions associated with supplementation with NEM[®]. The subjects' self-assessment diaries were reviewed and any discomfort or other adverse events were recorded and reported in accordance with applicable ICH Guidelines. Adverse events and serious adverse events were assessed by the clinical investigator at each study visit and followed until resolution, as necessary. Serious adverse events were required to be reported to the clinical investigator immediately.

2.6. Statistical Analysis

As this was an open-label study, a simple single-group sample size estimate [15] was performed for statistical power determination for a continuous variable. In the similar trial with NEM[®] conducted in Germany [11], the mean standard deviation for the study subjects for pain was 1.55 points. We hoped to be able to detect a 1.5 point difference from baseline within the 10-point Likert scale. Therefore a minimum of 18 subjects would need to be enrolled to have a 95% likelihood of detecting the expected improvement with a statistical power of 80%. Post-baseline statistical analyses were done as repeated measures Analysis of Variance (rm-ANOVA). Items found to have statistical significance with rm-ANOVA were then compared using a Wilcoxon test for dependent samples. Statistical significance was accepted at $p < 0.05$. Analysis of the primary outcome measure (the change from baseline in general pain levels) was conducted in the per protocol population. SPSS Statistics V19.0 was used for all statistical analyses [16].

3. Results

Patient recruitment began in May 2014 at a single clinical site in Italy and the final follow-up was conducted in July 2014. A total of twenty-five subjects between the ages of 43 and 81 were enrolled with osteoarthritis of the knee. Of these subjects, twenty (80%) were female and five (20%) were male. Of the twenty-five subjects with knee OA, 10 (40.0%) had bilateral incidence. Patient demographics are reported in Table 1. All twenty-five

Table 1. Patient demographics.^a

Age, yrs	69.4 ± 9.6
Sex	
Male (%)	5 (20)
Female (%)	20 (80)
Height, cm	165.8 ± 7.0
Weight, kg	71.0 ± 10.5
Body-mass Index	25.8 ± 3.2
Affected Joint	
Knee (l, r, bilateral)	25 (5, 10, 10)

a. Except where indicated otherwise, values are reported as mean ± standard deviation (SD) (n = 25). BMI was determined as weight in kilograms divided by height in meters squared.

subjects completed the one month study per the protocol. Compliance with the study treatment regimen was good.

A clinical comparison of valid subjects was carried out to obtain mean baseline scores for each of the ten questions from the subject questionnaire, as well as the 10-day and 30-day endpoints. Statistical analysis of the primary outcome measure revealed that supplementation with NEM[®] produced a significant treatment response from baseline at both 10 days and 30 days for composite pain (40.6% reduction, $p < 0.001$; 66.4% reduction, $p < 0.001$, respectively) (see **Figure 2(a)**). There was also a statistically significant concurrent reduction in analgesic use during the 30-day study period. At baseline, subjects consumed analgesic slightly less than one day per week on average, and this dropped 78.3% ($p = 0.017$) to 0.2 days through the 10-day endpoint. All 25 subjects consumed no analgesic through the final 3 weeks of the study ($p = 0.003$) (see **Figure 2(b)**). A significant treatment response from baseline was also observed for composite stiffness at both 10 days and 30 days (22.2% reduction, $p = 0.009$; 59.7% reduction, $p < 0.001$, respectively) (see **Figure 2(c)**). Supplementation with NEM[®] also produced a significant treatment response from baseline after 10 days when replying to Questions 1 - 5 & 7 - 8 (30.2% to 50.0% improvement) and at 30 days for all eight pain-related questions evaluated (50.9% to 78.9% improvement) (see **Table 2**). Treatment response fell shy of statistical significance for Question 6 at 10 days ($p = 0.190$). Similarly, a significant treatment response for stiffness was found at 10 days (Question 9) (27.7% improvement) but fell just shy of significance for Question 10 (15.4% improvement, $p = 0.069$). There was also a significant treatment response at 30 days for both stiffness-related questions (Q9 & Q10) (53.2% & 69.2%, respectively). There were no adverse events or serious adverse events reported during the study and the treatment was reported to be well tolerated by study participants.

4. Discussion

Osteoarthritis is very common in Italy with about one-third of the population having some form of the disease [2] [3]. This has a large impact on the quality of life of those afflicted with OA [17]. This open-label clinical trial was designed to evaluate the efficacy of NEM[®] as a natural arthritis treatment in an Italian population and to further validate the extension of the body of clinical evidence for NEM[®] from the United States to the general European population. The study demonstrated that NEM[®] is effective and safe for treating both pain and stiffness associated with osteoarthritis of the knee and results in the use of less analgesic medication.

Study subjects experienced relatively rapid (10 days) responses for both composite pain (40.6% improvement) and composite stiffness (22.2% improvement). By the end of the follow-up period (30 days) the mean response for composite pain and stiffness had increased substantially (66.4% improvement & 59.7% improvement, respectively). These results are quite similar to results from previous clinical studies of NEM[®] that were conducted in the U.S. [9] [10] and is a somewhat larger effect than what was found recently in a German population [11]. This difference may be a result of a small difference in mean pain at baseline between the two study populations (4.6 compared to 4.9 in Germany). Both studies showed statistically significant treatment effects at 10

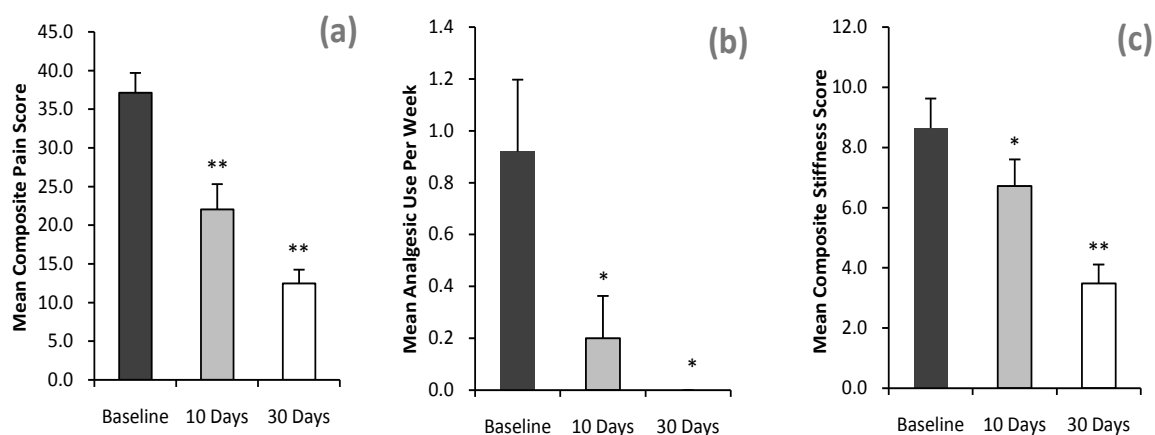


Figure 2. Mean composite pain score (a), mean analgesic user per week (b), and mean composite stiffness score (c) at baseline and 10 & 30 days of supplementation. Values are reported as means \pm standard deviation (SD) ($n = 25$). * $p < 0.05$, ** $p < 0.001$

Table 2. Mean values by question in an NEM-supplemented treatment group at baseline and 10 & 30 days post-treatment.

	Days Post-Treatment	Mean \pm SD	Percent Improvement	P-value ^a		Days Post-Treatment	Mean \pm SD	Percent Improvement	P-value ^a
Question 1	Baseline (n = 25)	5.1 \pm 0.3	-	-	Question 6	Baseline (n = 25)	2.9 \pm 0.6	-	-
	10 (n = 25)	3.1 \pm 0.5	39.2%	<0.001**		10 (n = 25)	2.2 \pm 0.6	24.1%	0.190
	30 (n = 25)	2.0 \pm 0.5	60.8%	<0.001**		30 (n = 25)	0.9 \pm 0.2	69.0%	0.002*
Question 2	Baseline (n = 25)	5.6 \pm 0.4	-	-	Question 7	Baseline (n = 25)	5.3 \pm 0.6	-	-
	10 (n = 25)	3.1 \pm 0.6	44.6%	<0.001**		10 (n = 25)	3.7 \pm 0.5	30.2%	0.005*
	30 (n = 25)	1.7 \pm 0.4	69.6%	<0.001**		30 (n = 25)	2.6 \pm 0.3	50.9%	<0.001**
Question 3	Baseline (n = 25)	3.2 \pm 0.6	-	-	Question 8	Baseline (n = 25)	3.8 \pm 0.6	-	-
	10 (n = 25)	1.6 \pm 0.5	50.0%	0.031**		10 (n = 25)	1.9 \pm 0.5	50.0%	0.006*
	30 (n = 25)	0.8 \pm 0.3	75.0%	<0.001**		30 (n = 25)	0.8 \pm 0.3	78.9%	<0.001**
Question 4	Baseline (n = 25)	5.6 \pm 0.4	-	-	Question 9	Baseline (n = 25)	4.7 \pm 0.6	-	-
	10 (n = 25)	3.5 \pm 0.4	37.5%	<0.001**		10 (n = 25)	3.4 \pm 0.5	27.7%	0.018*
	30 (n = 25)	1.9 \pm 0.3	66.1%	<0.001**		30 (n = 25)	2.2 \pm 0.4	53.2%	<0.001**
Question 5	Baseline (n = 25)	5.6 \pm 0.3	-	-	Question 10	Baseline (n = 25)	3.9 \pm 0.5	-	-
	10 (n = 25)	3.0 \pm 0.5	46.4%	<0.001**		10 (n = 25)	3.3 \pm 0.5	15.4%	0.069
	30 (n = 25)	1.8 \pm 0.4	67.9%	<0.001**		30 (n = 25)	1.2 \pm 0.3	69.2%	<0.001**

a. P-values were determined by Wilcoxon test for dependent samples following a statistically significant difference as determined by rm-ANOVA, and represent treatment versus baseline. * $p < 0.05$, ** $p < 0.001$.

days, so this is not too concerning. Study subjects also experienced large improvements in particular aspects of pain when reviewing the individual questions from the short-form questionnaire. For example, at 30 days there was a 75% improvement in pain while at rest (Question 3) and a 79% improvement in pain when putting on socks or pantyhose (Question 8). Likewise, pain when going up and down stairs (Question 2) and pain when getting in and out of a car, bathtub, etc. (Question 6) were both improved by nearly 70%. This broad treatment effect relating to numerous activities of daily living should have a profound impact on the subjects overall quality of life. This should also help them to remain active as they age, which is also important to other aspects of health (*i.e.* cardiovascular disease, neurodegenerative disease, etc.).

The safety profile for NEM[®] was again found to be excellent as there were no reports of adverse events or serious adverse events associated with treatment. This was comparable to the clinical trials conducted with NEM[®] previously [9]-[11]. No side effects from consuming NEM[®] have so far been identified, excluding the obvious egg allergy concern. This is very important in a disease like osteoarthritis that requires long-term treatment. The analgesics and NSAIDs normally used to treat such conditions are known to lead to gastric [7] and cardiovascular [8] complications which can considerably increase mortality in an elderly population.

The trial had a limited enrollment (25 subjects), however no subjects withdrew from the study and there was good treatment compliance. As the trial was also open-label, there is the obvious issue of the placebo effect. The inclusion of a placebo control would have provided greater clinical clarity, however it would have required a substantially larger study population. These limitations are minor when considering the totality of the available clinical evidence for the use of NEM[®] in joint and connective tissue disorders.

5. Conclusion

It is important for patients to have treatment options that are both safe and effective in managing chronic diseases such as osteoarthritis, especially in Italy where about one-third of the population is affected. The reporting of the results from this single-center, open-label clinical study demonstrates that NEM[®] brand eggshell membrane is a viable natural treatment option for the management of osteoarthritis of the knee. In this clinical study, NEM[®], 500 mg taken once daily, significantly reduced both composite pain and stiffness rapidly (10 days) and this effect continued to improve through 30 days. There was also a meaningful reduction in the amount of analgesic consumed on a weekly basis, which further enhanced patients' safety.

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References

- [1] Peat, G., McCarney, R. and Croft, P. (2001) Knee Pain and Osteoarthritis in Older Adults: A Review of Community Burden and Current Use of Primary Health Care. *Annals of the Rheumatic Diseases*, **60**, 91-97. <http://dx.doi.org/10.1136/ard.60.2.91>
- [2] Mannoni, A., Briganti, M.P., Di Bari, M., Ferrucci, L., Costanzo, S., Serni, U., *et al.* (2003) Epidemiological Profile of Symptomatic Osteoarthritis in Older Adults: A Population Based Study in Dicomano, Italy. *Annals of the Rheumatic Diseases*, **62**, 576-578. <http://dx.doi.org/10.1136/ard.62.6.576>
- [3] Schaap, L.A., Peeters, G.M.E.E., Dennison, E.M., Zambon, S., Nikolaus, T., Sanchez-Martinez, M., *et al.* (2011) European Project on Osteoarthritis (EPOSA): Methodological Challenges in Harmonization of Existing Data From Five European Population-based Cohorts on Aging. *BMC Musculoskeletal Disorders*, **12**, 272. <http://dx.doi.org/10.1186/1471-2474-12-272>
- [4] Altman, R. (1999) Ibuprofen, Acetaminophen and Placebo in Osteoarthritis of the Knee: A Six-Day Double-Blind Study [Abstract]. *Arthritis & Rheumatology*, **42**, S403.
- [5] Case, J.P., Baliunas, A.J. and Block, J.A. (2003) Lack of Efficacy of Acetaminophen in Treating Symptomatic Knee Osteoarthritis. *Archives of Internal Medicine*, **163**, 169-178. <http://dx.doi.org/10.1001/archinte.163.2.169>
- [6] Geba, G.P., Weaver, A.L., Polis, A.B., Dixon, M.E. and Schnitzer, T.J. (2002) Efficacy of Rofecoxib, Celecoxib, and Acetaminophen in Osteoarthritis of the Knee. *Journal of the American Medical Association*, **287**, 64-71. <http://dx.doi.org/10.1001/jama.287.1.64>
- [7] Laine, L. (1996) Nonsteroidal Anti-inflammatory Drug Gastropathy. *Gastrointestinal Endoscopy Clinics of North America*, **6**, 489-504.
- [8] Singh, G., Wu, O., Langhorne, P. and Madhok, R. (2006) Risk of Acute Myocardial Infarction with Nonselective Non-Steroidal Anti-Inflammatory Drugs: A Meta-Analysis. *Arthritis Research & Therapy*, **8**, R153-R162. <http://dx.doi.org/10.1186/ar2047>
- [9] Ruff, K.J., DeVore, D.P., Leu, M.D. and Robinson, M.A. (2009) Eggshell Membrane: A Possible New Natural Therapeutic for Joint and Connective Tissue Disorders. Results from Two Open-Label Human Clinical Studies. *Clinical Interventions in Aging*, **4**, 235-240. <http://dx.doi.org/10.2147/CIA.S5797>
- [10] Ruff, K.J., Winkler, A., Jackson, R.W., DeVore, D.P. and Ritz, B.W. (2009) Eggshell Membrane in the Treatment of Pain and Stiffness from Osteoarthritis of the Knee: A Randomized, Multicenter, Double-Blind, Placebo-Controlled Clinical Study. *Clinical Rheumatology*, **28**, 907-914. <http://dx.doi.org/10.1007/s10067-009-1173-4>
- [11] Danesch, U., Seybold, M., Rittinghausen, R., Treibel, W. and Bitterlich, N. (2014) NEM[®] Brand Eggshell Membrane Effective in the Treatment of Pain Associated with Knee and Hip Osteoarthritis: Results from a Six-Center, Open-Label German Clinical Study. *Journal of Arthritis*, **3**, 136. <http://dx.doi.org/10.4172/2167-7921.1000136>
- [12] Wong, M., Hendrix, M.J.C., von der Mark, K., Little, C. and Stern, R. (1984) Collagen in the Egg Shell Membranes of the Hen. *Developmental Biology*, **104**, 28-36. [http://dx.doi.org/10.1016/0012-1606\(84\)90033-2](http://dx.doi.org/10.1016/0012-1606(84)90033-2)
- [13] Baker, J.R. and Balch, D.A. (1962) A Study of the Organic Material of Hen's-Egg Shell. *Biochemical Journal*, **82**, 352-361. <http://dx.doi.org/10.1042/bj0820352>
- [14] Long, F.D., Adams, R.G. and DeVore, D.P. (2005) Preparation of Hyaluronic Acid from Eggshell Membrane. US Patent No. 6946551.
- [15] National Research Council (2003) Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. National Academies Press, Washington DC. https://grants.nih.gov/grants/olaw/National_Academies_Guidelines_for_Use_and_Care.pdf
- [16] IBM Corporation, SPSS Statistics. www.IBM.com
- [17] Cook, C., Pietrobon, R. and Hegedus, E. (2007) Osteoarthritis and the Impact on Quality of Life Health Indicators. *Rheumatology International*, **27**, 315-321. <http://dx.doi.org/10.1007/s00296-006-0269-2>

Effects of natural eggshell membrane (NEM) on monosodium iodoacetate-induced arthritis in rats*

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ABSTRACT

Purpose: The aim of this study is to investigate the anti-arthritis activities using natural eggshell membrane (NEM). **Methods:** NEM was administered at 52 mg/kg, 200 mg/kg, and 400 mg/kg to SD-Rat, where arthritis was induced by monosodium iodoacetate (MIA) at 3 mg. NO production in serum was measured using Griess reagent. Cytokines including IL-1 β , and IL-6 were measured by Luminex and PGE₂, MMP-2, MMP-9, TIMP-1, LTB₄, and hs-CRP were measured by ELISA. The cartilage of patella volume was examined, and 3-D high-resolution reconstructions of the cartilage of patella were obtained using a Micro-CT system. **Results:** Production of NO, IL-1 β , IL-6, PGE₂, MMP-2, MMP-9, TIMP-1, LTB₄, and hs-CRP in serum was decreased, respectively, in comparison with control. The cartilage of patella volume increased significantly. In addition, the NEM group showed a decrease in the cartilage of patella, synovial membrane, and transformation of fibrous tissue. **Conclusion:** The results for NEM showed significant anti-arthritis activity. These results may be developed as a raw material for new health food to ease the symptoms mentioned above.

KEY WORDS: osteoarthritis, anti-inflammatory, NEM, MIA, health food

Introduction

Osteoarthritis(OA), also called degenerative arthritis, is one of the most common types of arthritis. In most cases, osteoarthritis develops in the weight-bearing joints of the knees, characterizing local deformation of affected joint cartilage, joint swelling, excessive bone formation of the affected bone cartilage, transformation of joint. It is a disease causing the symptoms of

osteoarthritis, which tend to gradually develop, including repetitive joint pain and soreness, gradual motility disorder. In addition, the affected joints may also be stiff or creaky. The cause of osteoarthritis has not been investigated exactly yet, but many factors - such as genetics, age, injury, obesity - may play a role.

Women over the age of 50 are vulnerable to osteoarthritis showing 95% incidence rate

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under the age of 75. Recently, osteoarthritis has been treated focused on improving the symptoms by using medical treatment-such as anti-inflammatory analgesic drug and cartilage protection- and nondrug treatment. In the case of anti-inflammatory analgesic drug, it can cause the side effect on gastro-intestinal tract when taken for a long period and total joint replacement can also develop complications.

Taken all these together, the preference and demand on health functional food have increased for the treatment of osteoarthritis. However, the medical field has doubted the effects of bone-related health functional food. To maintain and develop the arthritis health functional food market, therefore, alternative needs to be concerned in developing health functional food for osteoarthritis and in this point of view, the need for studying the source of health functional food is increasing.

Eggshell membrane is the clear film lining of an eggshell having a function of letting oxygen pass and blocking invasion of bacteria. Eggshell membrane is one of the elements that make up the egg that occupies an important position as a complete food in the human diet. It is composed of 70% organic matter, 10% inorganic material and 20% water and 80% of the organic material constitutes the main component, and the rest is made of 2.3% of the fat and 3.4% carbohydrate.

However, of the elements that make up the egg, egg yolk, egg white is used as a raw material for processed foods and egg shell has been used as a raw material for a calcium supplement to play an important role in dental and skeletal formation. In contrast, in case of eggshell, since the utilization is not high, it is classified as waste.

As a pre-study of the animal model in eggshell was LPS-induced inflammatory experiment and reported the efficacy of anti-arthritis by reducing the inflammatory cytokines and chemokines level. According to this basis, eggshell membrane is considered to be

efficacious for osteoarthritis yet the mechanism and efficacy study of the anti-arthritis for this situation is minimal. The purpose of this study was in accordance with previous studies, in osteoarthritis-induced animal model, through the study of arthritis-inducing factor, the purpose of this study was to improve the prevention and application in material development for new eco-friendly and effective supplements.

Methods

Materials and Methods

NEM (Natural eggshell membrane manufactured by ESM Technologies, LLC, USA) was prepared by Ju Yeong NS Co., Ltd., Seoul, Republic of Korea. To investigate the effect of prevention and treatment on arthritis, 2 weeks before inducing osteoarthritis by MIA (monosodium iodoacetate), NEM was administered with 2 ml orally at a dose of 52 mg/kg, 200 mg/kg, and 400 mg/kg daily, once a day at 10 A.M. and the same volume of distilled water was used as a vehicle control group. After 2 weeks of oral administration, osteoarthritis was induced and the same amount at the same concentration of NEM was orally administered for 4 weeks.

Animals, Care and Diet

Male SD-Rats (170-200 g) at 6 weeks of age were obtained from RaonBio (Seoul, Korea) and animals were housed in cages under standard experimental condition ($22\pm 2^{\circ}\text{C}$; $55\pm 15\%$ humidity; 12-hour light/dark cycle) and had access to standard rat chow (Purina, Nestle). Animals were used in experiment after having 2 weeks of adjustment period. All animal procedures were approved by the Daejeon University Animal Care Committee (Approved number-DJUARB2014-023) and were performed in accordance with the guidelines of the National Institutes of Health.

Rats were anesthetized with the mixture of Zoletil 0.5 ml and rompun 0.1 ml. Arthritis was induced after shaving the knee of the rats. Rats were given a single intra-articular injection of 50 μ l at a dose of 3 mg/ml MIA (Sigma-Aldrich Corporation, MO, USA) through the right knee. MIA was dissolved in 0.9% saline. 35 Rats were divided into 5 groups; normal group without MIA-induction, MIA-induced arthritis (administered distilled water) control group, MIA-induced arthritis with administration of NEM experimental group, etc.

Blood Collection

After the final end of experiment using a heart puncture, heart blood was collected 10 ml of blood into the syringe tube. After 30 minutes at room temperature and hardened by centrifugation at 3,000 rpm for 15 minutes, to separate the serum, hs-CRP was measured by referral to the Research Institute of Seoul (Seoul, Korea).

Cytokine Measurement

The IL-1 β and IL-6 in serum separated from the blood was measured to check the inflammatory cytokines and arthritis-inducing factor in the serum and were measured using Mouse cytokine milliplex map immunoassay kit (Millipore, MA, USA) by Luminex (Millipore, MA, USA). NO (Intron Biotechnology, Suwon, Korea), PGE₂ (R&D system, Minneapolis, USA), MMP-2 (R&D system, Minneapolis, USA), MMP-9 (R&D system, Minneapolis, USA), LTB₄ (R&D system, Minneapolis, USA), Rat CTX-2 ELISA kit, (Biotang, MA, USA), Rat COMP ELISA kit (AnaMar Medical AB, Lund, Sweden) were measured by ELISA reader (Molecular Devices, CA, USA).

Micro-CT Arthrography Measurement

Micro-CT measurements were performed at Yonsei University (Wonju, Korea), A hex Briggs (HEXABRICS 320) injected into the tail vein using a micro-CT-arthrography and the amount of knee joint cartilage (cartilage volume) were measured and analyzed.

Immunohistochemistry

After shooting Micro-CT, the knees were cut and decalcified in formalin solution containing 10% EDTA. Using radiographic technique, after identifying the result of decalcification, joint tissues were put in paraffin wax and fixed and then performed coronal section. After the procedure of decalcification, the tissue fixed by paraffin was cut into 7 μ m, and stained by Hematoxylin and Eosin (H&E) and Safranin-O to observe the tissue.

Statistical Analysis

SPSS 11.0 (statistical package for social sciences, Version 10.0, Chicago, USA) was used for all statistical analysis. using unpaired student's T-test and statistical significance was accepted at an α value of $p < 0.05$, $p < 0.01$ or $p < 0.001$.

Results

Effect of NEM on NO and PGE₂

Nitric oxide in the blood (nitric oxide, NO) measurement of the production are as follows:. The normal group was 8.1 ± 2.5 μ M, the control group showed a 16.2 ± 2.2 μ M and, NEM treated group showed 11.8 ± 4.6 μ M in the 52 mg/kg, 7.0 ± 2.4 μ M in the 200 mg/kg, and 5.3 ± 2.9 μ M in the 400 mg/kg showing a tendency to gradually decrease as the concentration of NO in NEM group compared to the control group. In particular, it showed a significant reduction in the concentration of the group 400 mg/kg and 200 mg/kg (Fig. 1). In addition, the result of prostaglandins-E₂ (ProstaGlandin-E₂, PGE₂) measurement of the production was 1550.5 ± 172.8 pg/ml in normal group, 2482.9 ± 315.4 pg/ml in control group and NEM treated group was 1802.6 ± 540.6 pg/ml in 52 mg/kg, 1518.9 ± 411.8 pg/ml in 200 mg/kg, and 989.8 ± 435.6 pg/ml in 400 mg/kg showing a significant reduction in all concentrations (Fig. 2).

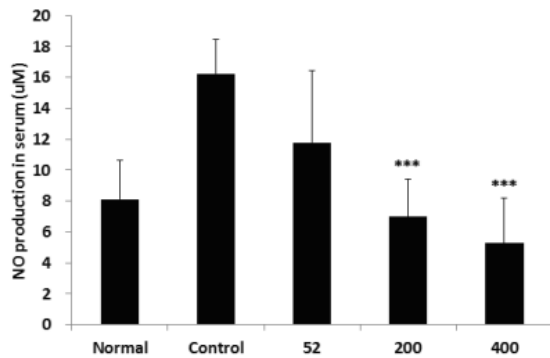


Fig. 1. Effects of NEM on levels of NO in the serum of MIA-induced osteoarthritis rats. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test (** $p < 0.001$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

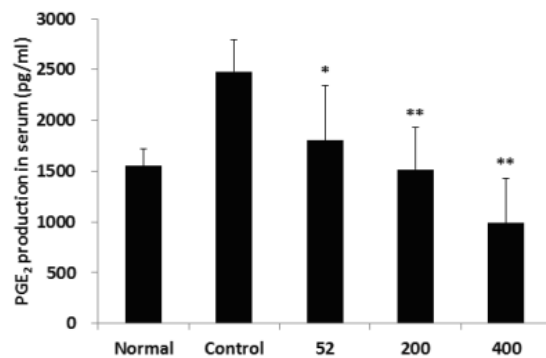


Fig. 2. Effects of NEM on levels of PGE₂ in the serum of MIA-induced osteoarthritis rat. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test (* $p < 0.05$, ** $p < 0.01$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

Effect of NEM on cytokine levels

In the measurement of the IL-1 β production in the blood, normal group showed 40.50 ± 2.52 pg/ml, the control group was found to be 72.50 ± 8.23 pg/ml, NEM treated group was found to be 52.02 ± 10.20 pg/ml in 52 mg/kg, 43.61 ± 6.83 pg/ml in 200 mg/kg, and 37.50 ± 7.19 pg/ml in 400 mg/kg showing a dose-dependent manner with significant reduction (Fig. 3A). In the case of IL-6 production, the control group was $61.75 \pm$

2.63 pg/ml, the control group was found to be 136.00 ± 13.37 pg/ml. NEM treated group was 121.50 ± 7.72 pg/ml at 52 mg/kg, 113.50 ± 14.27 pg/ml in 200 mg/kg, and 86.50 ± 16.03 pg/ml in 400 mg/kg showing reduction and in particular it showed a significant reduction in the group of 400 mg/kg (Fig. 3B).

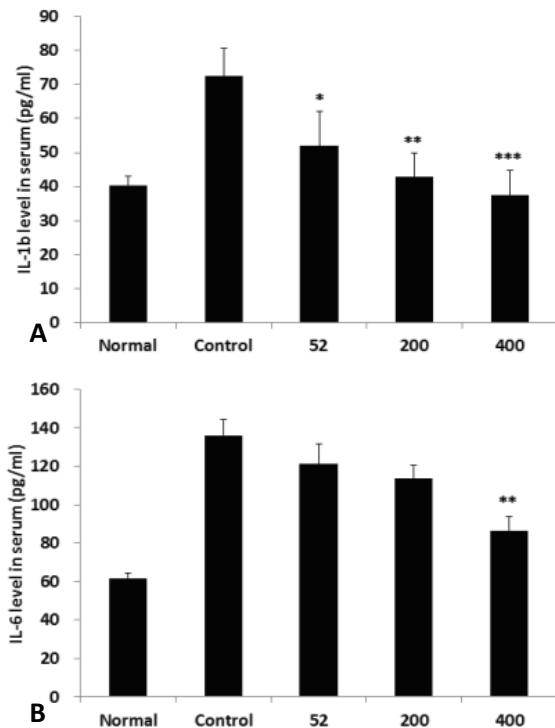


Fig. 3. Effects of NEM on levels of IL-1 β in the serum of MIA-induced osteoarthritis rats. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

Effect of NEM on hs-CRP

As a result of high sensitivity C-reactive protein in the blood (hs-CRP) measurement, normal group was 0.21 ± 0.04 mg/L, control group was 0.61 ± 0.07 mg/L, NEM-treated group showed 0.25 ± 0.07 mg/L in 52 mg/kg, 0.24 ± 0.09 mg/L in 200 mg/kg, 0.22 ± 0.05 mg/L in 400 mg/kg showing a significant reduction in all concentrations (Fig. 4).

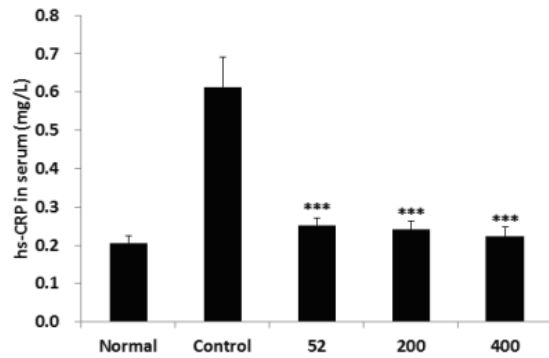


Fig. 4. Effects of NEM on levels of hs-CRP in the serum of MIA-induced osteoarthritis rats. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test (** $p < 0.001$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

Effect of NEM on MMP's and LTB₄

Among the matrix proteases in the blood (matrix metalloproteinase, MMP), the results of MMP-2 and MMP-9 are as follows. In case of MMP-2, the normal group was 0.45 ± 0.04 ng/ml, and control group was found to be 0.85 ± 0.08 ng/ml. NEM-treated group was 0.71 ± 0.12 ng/ml in 52 mg/kg, 0.66 ± 0.10 ng/ml in 200 mg/kg, 0.59 ± 0.08 ng/ml in 400 mg/kg showing a concentration-dependent reduction (Fig. 5A). In the case of MMP-9 production, normal group was 0.31 ± 0.06 ng/ml, control group was 0.65 ± 0.06 ng/ml, and NEM-treated group was 0.44 ± 0.15 ng/ml in 52 mg/kg, 0.36 ± 0.11 ng/ml at 200 mg/kg, and 0.36 ± 0.09 ng/ml at 400 mg/kg showing a concentration-dependent reduction (Fig. 5B). As a result of measuring the production of leukotriene B₄ (LTB₄), normal group was 463.6 ± 72.3 pg/ml, control group was 1488.5 ± 242.5 pg/ml, NEM-treated group was 939.9 ± 203.6 pg/ml at 52 mg/kg, 833.1 ± 376.6 pg/ml at 200 mg/kg, 794.5 ± 275.6 pg/ml at 400 mg/kg showing a significant reduction in all doses (Fig. 6).

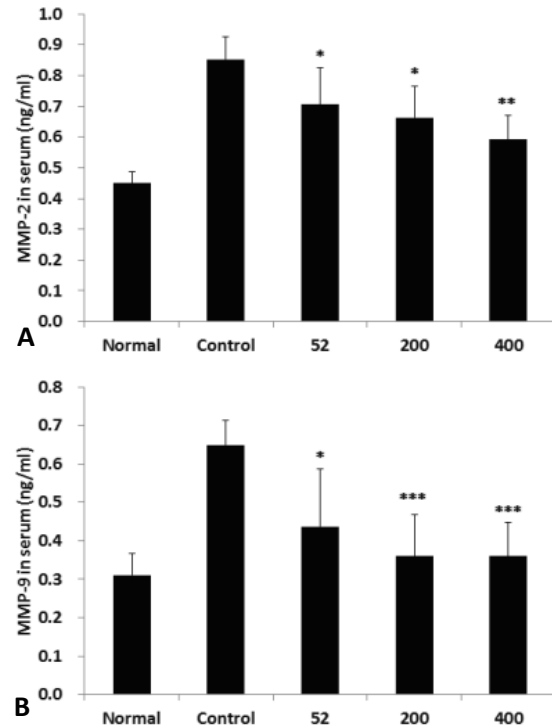


Fig. 5. Effects of NEM on levels of MMP-2 in the serum of MIA-induced osteoarthritis rats. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test (* $p < 0.05$, ** $p < 0.01$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

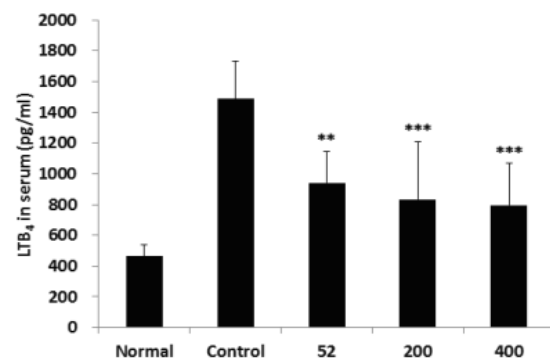


Fig. 6. Effects of NEM on levels of LTB₄ in the serum of MIA-induced osteoarthritis rats. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test (** $p < 0.01$, *** $p < 0.001$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

Effect of NEM on cartilage damage

The measurement of the cartilage damage in the blood is as follows. In COMP (Cartilage Oligomeric Matrix Protein) production, normal group was 90.2 ± 12.9 ng/ml, control group was 134.4 ± 18.1 ng/ml, NEM-treated group was 115.2 ± 17.0 ng/ml at 52 mg, 107.8 ± 18.6 ng/ml at 200 mg, and 101.9 ± 15.5 ng/ml at 400 mg ($*p < 0.05$) (Fig. 7). Also, as a result of CTX-II (C-telopeptide of type II collagen) production, normal group was 1.8 ± 1.3 pg/ml, control group was 273.7 ± 49.2 pg/ml, NEM-treated group was 49.5 ± 7.7 pg/ml at 52 mg ($**p < 0.01$), 38.7 ± 11.5 pg/ml at 200 mg ($**p < 0.01$), and 73.2 ± 22.6 pg/ml at 400 mg ($**p < 0.01$) showing a significant decrease at all doses (Fig. 8).

Effect of NEM on knee joint pathology

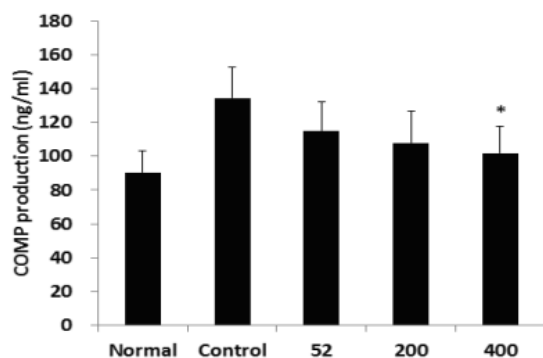


Fig. 7. Effects of NEM on levels of COMP in the serum of MIA-induced osteoarthritis rats. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test ($*p < 0.05$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

To determine the changes and infiltration of inflammatory cells in the synovial cells of knee joints, H&E staining was used. While the control group induced osteoarthritis resulting in deformation of the cartilage and synovial membrane and fibrous tissue remarkably than the normal group, deformation of the cartilage and synovial tissue fibers in NEM treated group was

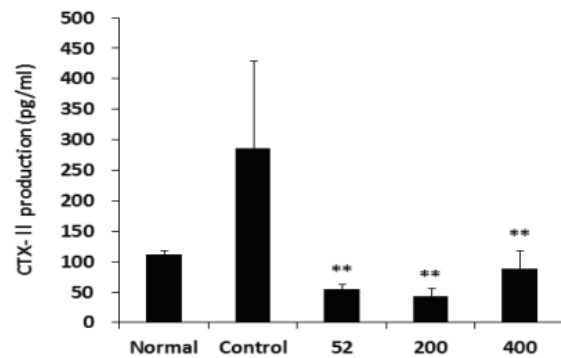


Fig. 8. Effects of NEM on levels of CTX-II in the serum of MIA-induced osteoarthritis rats. The results are expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test ($**p < 0.01$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

found to be suppressed than in the control group (Fig. 9A). In addition, knee cartilage proteoglycan layers stained with Safranin-O were tested to confirm the damage of cartilage. As a result, the control group was induced osteoarthritis, leading to the transformation of the joint tissue and the cartilage cells next to synovial membrane was found to be severely damaged. The deformation of the joint tissue in NEM group was suppressed compared with the control, and damage of the cartilage cells was also found to be inhibited (Fig. 9B).

Effect of NEM on cartilage volume

As a result of imaging by using a micro CT-arthrography of the amount of knee joint cartilage, It was confirmed that the cartilage in the normal group was more reduced compared to the control group. In NEM treated group, the amount of cartilage was more preserved than that of control group (Fig. 10A). The results of measuring the amount of the area of preserved cartilage were 0.63 ± 0.10 mm³ in normal group, 0.20 ± 0.08 mm³ in control group and NEM treated group showed 0.45 ± 0.12 mm³ at 52

mg/kg, $0.39 \pm 0.08 \text{ mm}^3$ at 200 mg/kg, and $0.37 \pm 0.11 \text{ mm}^3$ at 400 mg/kg (Fig. 10B).

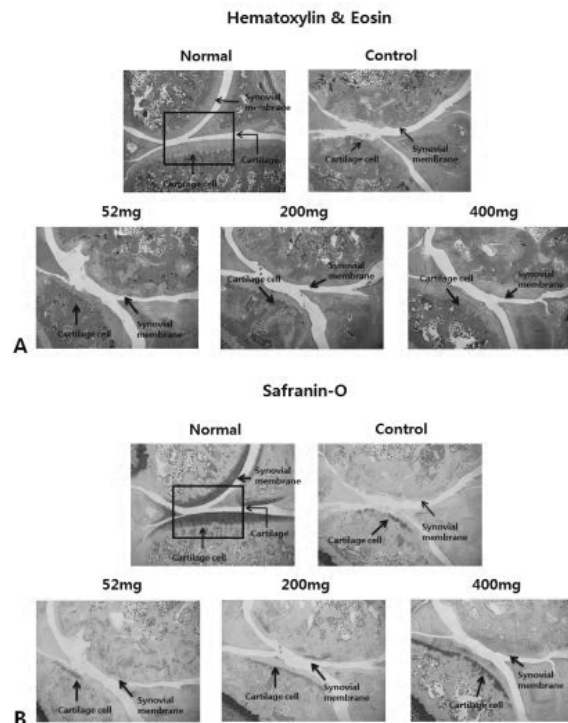


Fig. 9. Effects of NEM on joint pathology (Hematoxylin & Eosin staining) and (Safranin-O) from joint tissue of MIA-induced osteoarthritis rats. Normal group shows the presence of slightly thickened synovium (arrow) Control group shows isolated areas with chronic inflammation (arrow). NEM treated group shows signs of tissue integrity with a thick layer of cartilage compared with control group (arrow). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

Discussion

In recent years, 65 years or older population is increasing by an average life span increased with the development of medicine and nutrition. This causes diabetes, high blood pressure, Alzheimer's disease, and a number of studies on the age-related disease such as osteoarthritis are being actively investigated. Among these diseases, osteoarthritis is a disease that its prevalence increases proportionally with age. However, since some view osteoarthritis as a degenerative phenomenon so osteoarthritis has

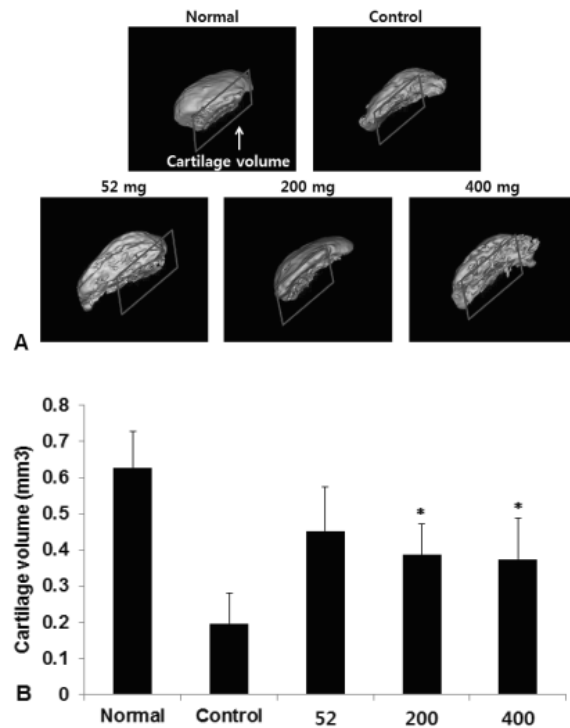


Fig. 10. Effects of NEM on imaging of cartilage degeneration using micro CT-arthrography in joint tissue of MIA-induced osteoarthritis rats. The results were expressed as mean \pm S.D. from 7 osteoarthritis rats. Statistically significant value compared with control group by unpaired student's t-test (* $p < 0.05$). Normal, Normal SD-rat group; Control, MIA-induced osteoarthritis group; 52, MIA-induced osteoarthritis group + NEM 52 mg/kg; 200, MIA-induced osteoarthritis group + NEM 200 mg/kg; 400, MIA-induced osteoarthritis group + NEM 400 mg/kg.

been overlooked in the treatment of aging and disease management. The current treatment for osteoarthritis is nonpharmacologic therapy, medication, surgery, etc but these all hang reactivated when stop taken. In clinical research, the effects of those are inadequate or last for a short time so the study on treatment for osteoarthritis is still ongoing. Accordingly, it is necessary to study the prevention and improvement of osteoarthritis in serving as a new osteoarthritis drug development.

Natural Eggshell Membrane, NEM, used in this study is an environmentally-friendly and effective material proved by a variety of research and the number of experiments for osteoarthritis is currently ongoing. According to the results

reported up to now, it has been found that NEM is an effective substance in pain relief in clinical osteoarthritis and reduction of cytokines in osteoarthritis-induced animal experiments.

Therefore, in accordance with previous studies, this study was to determine the efficacy of NEM by studying the inducer of osteoarthritis in osteoarthritis-induced animal model. First, to identify the effects of NEM on the prevention and improvement of osteoarthritis, from the 2 weeks before the MIA-induced osteoarthritis to 4 weeks after the MIA-induced osteoarthritis, NEM were orally administered to the animals at all concentrations and then the OA-induced factors, histopathological examination and micro-CT were tested through the blood test, joint and cartilage.

When divided by the factor associated with the pathogenesis of osteoarthritis, proteolytic enzymes, cytokines, and the nitric oxide are known as major factors. Among these, NO, one of the inflammatory mediators of vascular system plays an important role in hypertension, neurotransmission, and the immune system and is produced excessively during the process of various diseases such as rheumatoid arthritis and osteoarthritis. And the excessive production of NO, resulting in damage to the tissue and nerves, gene mutation, edema by increasing vascular permeability, leads to excessive inflammatory responses. Further, if inflammation occurs in the human body, PGE₂, the most important substance in the pathogenesis of inflammation, is produced very quickly by COX-2 in macrophages. While NO plays many roles in the early stages of white blood cells movement to the site of inflammation, PGE₂ acts mainly fever and pain that appears later and develops osteophytes resulting in an abnormal bone growth and pain frequently in osteoarthritis. In this study, the measurement of NO in NEM-treated group showed significant reduction in both 200 and 400 (mg/kg) groups, and the measurement of PGE₂ exhibited a

significant reduction at all treatment concentrations.

Cytokines regulate the inflammatory response by Th1 cells. Th1 cells promote inflammatory cytokines such as IL-1, IL-6 by activating monocytes and synoviocytes and also promote localized bone destruction by activating B cells leading to the differentiation of osteoclasts. Among these cytokines, IL-1 β is the production of other inflammatory mediators, including PGE₂ and NO from the cartilage and synovial cells, and by stimulating the expression of MMPs, it is known as a powerful cytokine that causes the degradation of cartilage in osteoarthritis. IL-6 is a pro-inflammatory cytokine secreted by the inflammatory response, and regulates the immune response, hematopoiesis, inflammation secreted by helper T cells, macrophages, mast cells, neutrophils, epithelial cells, fibroblasts, etc. Katherine also stated that IL-6 shows higher activity in the synovial fluid secreted by the blood vessels in the synovium and joint tissues than that of serum indicating the important role of IL-6 in osteoarthritis. In this study, in NEM treated group compared to the control IL-1 β , IL-6 was reduced significantly, especially in a concentration 400mg/kg.

CRP (Reactive Protein) is a typical acute phase reaction molecule that increases or decreases in response to inflammation or tissue damage. For this reason, CRP, one of the proteins called acute phase reactants, may be used to monitor the change of infectious diseases and inflammation. Hs-CRP test, used in this experiment, is a measurement which can more accurately detect the low levels of protein than standard CRP test found that it is related to the concentration of IL-6 in serum by Madhok et al. The measurement of the hs-CRP in this study, NEM treated group showed a significant reduction in all treatment concentrations.

MMPs (matrix metalloproteinases) play an important role in recovery in tissue, angiogenesis,

forming the shape of tissue, and are secreted by fibroblasts, keratinocytes, and macrophages. When osteoarthritis occurs, the protease MMPs and tissue inhibitor of MMPs (TIMP) are activated by antagonism of metalloproteinases system and break down proteoglycan. LTB_4 influences on bone metabolism and increases in rheumatoid arthritis, periodontal bone loss such as inflammatory diseases, so the control of MMPs and LTB_4 activities is important in improving osteoarthritis. In this study, NEM-treated group showed a decrease in significance at all concentrations for MMPs and LTB_4 . COMP (Cartilage Oligomeric Matrix Protein), cartilage damage indicator, is a protein component that is included in the cartilage, and the amount of blood COMP is being used to diagnose the conditions of cartilage, the degenerative condition, damage and progression. Further, the CTX- II (C-telopeptide of type II collagen) test which measures the wear amount of cartilage through the blood and urine, is one of the major biomarkers for osteoarthritis evaluation. When cartilage is worn by osteoarthritis, a specific protein is released and it is found that the higher the value of it, the faster OA progresses. In this study, NEM group showed a significant reduction in the COMP and CTX- II test. Analyzing the above results, NEM reduces MMPs and LTB_4 , osteoarthritis inducers, through the reduced efficacy of cytokines, nitric oxide and inflammatory mediators and it was confirmed to be affecting the COMP and CTX- II. To determine whether the results of the blood tests affect such tissue, joints, cartilage, etc., as a result of Hematoxylin & Eosin staining. While the control group was induced osteoarthritis causing significant deformation of cartilage, synovial membrane and the fibers more than the normal group, the deformation of fibrous tissue, cartilage, synovial membrane in NEM-treated group compared to the control group were

inhibited. In addition, the result of Safranin-O staining showed that the joint tissue of control group was modified and the cartilage in the synovial was severely damaged because of osteoarthritis. In contrast, NEM group showed little deformation of joint tissue and inhibition of damage of cartilage cell. Finally, and using micro-CT-arthrography, analyzing the amount of the knee joint cartilage (cartilage volume) and the area of the cartilage, the cartilage volume and the area of the cartilage in NEM-treated group increased compared to the control group.

Conclusion

Taken all these results together, the NEM-treated group protects the cartilage and joint tissue by reducing the osteoarthritis inducers and decreased bone destruction. Therefore, if further systematic clinical studies and experiments are conducted in-depth, it can be utilized as an environmentally-friendly and effective health functional food ingredient.

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References

1. Korean College of Rheumatology, KCR Textbook of rheumatology, 1st revision, Seoul; 2014.
2. The Society Korean Medicine Rehabilitation, Oriental Rehabilitation Medicine, 1st revision, Seoul; 2006.
3. Yoo MC. The latest trend in treating arthritis. *J Muscle Joint Health* 1995; 2(2): 227-229.
4. Auw Yang KG, Saris DBF, Dhert WJA, Verbout AJ. Osteoarthritis of the knee: current treatment options and future directions. *Current Orthopaedics* 2004; 18(4): 311-320.
5. Yang CH. Gastrointestinal disorders associated with non-steroidal anti-inflammatory drugs (NSAIDs). *DongGuk Med Rev* 2003; 10(2): 190-199.
6. Han JD, Shin JH, Hwang HC, Kim DY, Jo WH, Lim KI. Interaction of cytokines in osteolysis. *Journal of Korean orthopaedic research society* 2000; 3(2): 125-132.

7. Lee JW, Do JH. Market trend of health functional food and the prospect of ginseng market. *J Ginseng Res* 2005; 29(4): 206-214.
8. Kim HK. Current status and prospect of nutraceuticals. *Food Industry and Nutrition* 2004; 9(1): 1-14.
9. Leach RM, Rucker RB, Dyke GPV. Egg shell membrane protein: A non elastin desmosine/iso-desmosine containing protein. *Arch. Biochem. Biophys* 1981; 207(2): 353-359
10. Okubo T, Akachi S, Hatta H. Structure of hen eggs and physiology of egg laying. In: Takehiko Yamamoto, Lekh Raj Juneja, Hajime Hatta, Mujo Kim, editor. *Hen eggs: Their Basic and Applied Science*. MA: CRC Press LLC; 1997. p.1-12.
11. Jeon TW, Park KM. Functional properties of Egg Shell Membrane Hydrolysate as a Food Material. *Korean J. Food SCI. ANI. Resour* 2002; 22(3): 267-273
12. Ruff, KJ. DeVore, DP. Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Modern Research in Inflammation* 2014; 3(1): 19-25
13. Ruff, KJ, Winkler A, Jackson RW, DeVore DP, Ritz, BW. Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clinical rheumatology* 2009; 28(8): 907-914.
14. Jang YO. Pathogenesis of osteoarthritis. *Diagnosis and Treatment* 2007; 27(4): 395-399.
15. Park YK, Kim CS. The therapeutic effect of *Achyranthis Radix* on the collagen-induced arthritis in mice. *The Korea Journal of Herbolgy* 2010; 25(4): 129-135.
16. Wu W, Xu X, Dai Y, Xia L. Therapeutic effect of the saponin fraction from *Clematis chinensis* Osbeck roots on osteoarthritis induced by monosodium iodoacetate through protecting articular cartilage. *Phytother Res* 2010; 24(4): 538-546.
17. Wesche-Soldato DE, Swan RZ, Chung CS, Ayala A. The apoptotic pathway as a therapeutic target in sepsis. *Curr Drug Targets* 2007; 8(4): 493-500.
18. Herrington C, Hall PA. Molecular and cellular themes in inflammation and immunology. *J Pathol* 2008; 214(2): 123-125.
19. Campo GM, Avenoso A, Campo S, D'Ascola A, Traina P, Samà D, Calatroni A. Glycosaminoglycans modulate inflammation and apoptosis in LPS-treated chondrocytes. *J Cell Biochem* 2009; 106(1): 83-92.
20. Clancy RM, Amin AR, Abramson SB. The role of nitric oxide in inflammation and immunity. *Arthritis Rheum* 1998; 41(7): 1141-1151.
21. Rice-Evance C, Miller N, Paganga G. Antioxidant properties of phenolic compounds. *Trends Plant Sci* 1997; 2(4): 152-159.
22. Chang ST, Wu JH, Wang SY, Kang PL, Yang NS, Shyur LF. Antioxidant activity of extracts from *Acacia confusa* bark and heartwood. *J Agric Food Chem* 2001; 49(7): 3420-3424.
23. Hardy MM, Seibert K, Manning PT, Currie MG, Woerner BM, Edwards D, Koki A, Tripp CS. Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. *Arthritis Rheum* 2002; 46(7): 1789-1803.
24. Okamoto H, Hoshi D, Kiire A, Yamanaka H, Kamatani N. Molecular targets of rheumatoid arthritis. *Inflamm Allergy Drug Targets* 2008; 7(1): 53-66.
25. Scheinecker C, Redlich K, Smolen JS. Cytokines as therapeutic targets: advances and limitations. *Immunity* 2008; 28(4): 440-444.
26. Ji JD. Cytokines in rheumatoid arthritis. *Hanyang Med Rev* 2005; 25(2): 43-52.
27. Hulejova H, Baresova V, Klezl Z, Polanska M, Adam M, Senolt L. Increased level of cytokines and matrix metalloproteinases in osteoarthritic subchondral bone. *Cytokine* 2007; 38(3): 151-156.
28. LeGrand A, Fermor B, Fink C, Pisetsky DS, Weinberg JB, Vail TP, Guilak F. Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci. *Arthritis Rheum* 2001; 44(9): 2078-2083.
29. Tak MJ, Tak MR, Kang KH, Ko WS, Yoon HJ. The Inhibitory Effects of Yang Geouk San Hwa-Tang on LPS-stimulated inflammation in RAW264.7 macrophage cells. *J Korean Oriental Med Ophthalmol Otolaryngol Dermatol* 2010; 23(1): 118-134.
30. Katherine L, Manson TL, Moor JM. Cytokine level in synovial fluid and serum of patient with juvenile rheumatoid arthritis. *J Rheumatol* 1994; 21(12): 2359-2363.
31. Yeo HJ, Lee JH, Lee HJ, Byun KS, Im HJ, Kim MJ. Microvascular Findings in Patients with Rheumatoid Arthritis-Assessed, Using Fundus Photography and Fluorescein Angiography. *Journal of Rheumatic Diseases* 2013; 20(4): 231-237.
32. Madhok R, Crilly A, Watson J, Capell HA. Serum interleukin 6 levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity. *Ann Rheum Dis* 1993; 52(3): 232-234.
33. Reijman M, Hazes JMW, Bierma-Zeinstra SMA, Koes BW, Christgau S, Christiansen C, Pols HAP. A new marker for osteoarthritis: cross-sectional and longitudinal approach. *Arthritis & Rheumatism* 2004; 50(8): 2471-2478.
34. Garnero P, Peterfy C, Zaim S, Schoenharting M. Bone marrow abnormalities on magnetic resonance imaging are associated with type II collagen degradation in knee osteoarthritis: a three-month longitudinal study. *Arthritis & Rheumatism* 2005; 52(9): 2822-2829.
35. Elsaid KA, Chichester CO. Collagen markers in early arthritic diseases. *Clinica Chimica Acta* 2006; 365(1): 68-77.
36. Bresnihan B. Pathogenesis of joint damage in rheumatoid arthritis. *J Rheumatol* 1999; 26(3): 717-719.
37. Peters-Golden M, Henderson WR Jr. Leukotriene. *N Engl J Med* 2007; 357(18): 1841-1854.

Eggshell membrane hydrolyzates activate NF- κ B in vitro: possible implications for in vivo efficacy

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Purpose: Eggshell membrane (ESM) has been shown to contain naturally occurring bioactive components, and biological activities such as reducing proinflammatory cytokines, liver fibrosis, and joint pain in osteoarthritis sufferers have also been reported for ESM matrix as a whole. Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) is a signaling protein found in the cytoplasm of nearly all human and animal cell types and is a primary regulator of immune function. The studies reported herein were designed to investigate the possible role that NF- κ B activity might play in the reported biological activities of ESM.

Methods: Three ESM hydrolyzates produced via fermentation, enzymatic, or chemical hydrolysis were evaluated in vitro in either human peripheral blood mononuclear cell or THP-1 (human leukemic monocyte) cell cultures for NF- κ B activity following 4-hour exposure. The hydrolyzates were compared with untreated control cells or cells incubated with lipopolysaccharide or ascorbic acid. The source of ESM activity was also evaluated.

Results: NF- κ B levels were increased above levels found in untreated cells at all three dilutions (1:100, 1:1,000, and 1:10,000) for the fermentation hydrolyzate of ESM (ESM-FH) ($P=0.021$, $P=0.020$, $P=0.009$, respectively) in peripheral blood mononuclear cells. The enzymatic hydrolyzate of ESM (ESM-EH) also produced statistically significant levels of activated NF- κ B at the 1:100 and 1:1,000 dilutions ($P=0.004$, $P=0.006$, respectively) but fell just shy of significance at the 1:10,000 dilution ($P=0.073$). Similarly, ESM-FH ($P=0.021$, $P=0.002$) and ESM-EH ($P=0.007$, $P=0.007$) activated NF- κ B in THP-1 cells at 1:1,000 and 1:10,000 dilutions, respectively. The chemical hydrolyzate of ESM (ESM-CH) showed statistically significant levels of activation at the 1:1,000 dilution ($P=0.005$) but failed to differ from untreated cells at the 1:10,000 dilution ($P=0.193$) in THP-1 cells.

Conclusion: Results from our studies provide evidence that ESM hydrolyzates significantly activate NF- κ B, and the source of this activity was investigated to confirm that it is inherent to ESM and not derived from bacterial contamination. Based on our findings, we propose a plausible hypothesis as to how increased NF- κ B activity might translate into the in vivo efficacy that has been observed with ESM via an “oral tolerance” mechanism.

Keywords: eggshell membrane, NF- κ B, lipopolysaccharide, polymyxin B, lipoprotein lipase, hydrolyzate

Introduction

Eggshell membrane (ESM), found between the calcified shell and the albumin in chicken eggs, is primarily composed of fibrous proteins such as collagen type I¹ that form the mesh-like structure of the bilayered material. ESMs have also been shown to contain other bioactive components, namely glycosaminoglycans (ie, dermatan sulfate,² chondroitin sulfate,² and hyaluronic acid³). ESM is known to reduce the

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expression of various proinflammatory cytokines, including interleukin-1-beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) both in vitro⁴ and in vivo.⁵ A proprietary form of ESM, commercially available as the branded product NEM[®], has demonstrated safety and efficacy in multiple clinical trials in relieving joint pain and stiffness in humans with osteoarthritis^{6–8} and has been investigated for similar uses in various species of animals.^{9,10} ESM is also reported to have possible beneficial effects in liver fibrosis in rats through regulating gene expression.¹¹

Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) is a protein complex found in the cytoplasm of nearly all human and animal cell types; the deoxyribonucleic acid (DNA)-binding activities of this family of transcription factors were first reported in 1986.¹² Once activated and translocated to the nucleus, NF- κ B regulates the expression of multiple genes involved in the acute response to negative or harmful external cellular stimuli. Active NF- κ B is a heterodimeric protein that activates DNA sequences known collectively as κ B sites (see¹³ for a detailed overview). There are two well-accepted paths to activation of NF- κ B: the canonical or classical pathway and the noncanonical or alternative pathway. In either pathway (canonical or non-canonical), the inhibitor of κ B kinase complex is activated when signaling molecules bind to cell surface receptors. Bacterial or viral antigens,^{14,15} various cytokines (eg, IL-1 β , TNF- α),^{16,17} oxidative stress,^{18,19} ultraviolet irradiation,^{20,21} and free radicals^{22,23} are just some of the stimuli known to trigger activation of one or the other of the NF- κ B pathways, either through direct binding to cell surface receptors or by causing other signaling molecules to be formed, which then bind to the receptors.

NF- κ B was initially investigated for its critical role in regulating the immune response to infection. However, in the decades since its discovery, the dysregulation of NF- κ B has been associated with numerous classical inflammatory diseases such as sepsis, asthma, rheumatoid arthritis, and inflammatory bowel disease (see^{24–27} for a review). Interestingly, a number of diseases that are not obviously inflammatory in nature are also associated with NF- κ B dysregulation, including atherosclerosis,^{28,29} Alzheimer's disease,^{30,31} multiple sclerosis,^{32,33} diabetes,^{34,35} and various cancers.^{36–38}

Because of its fundamental importance in immune system function and inflammatory diseases, the studies reported herein were designed to investigate the possible role that NF- κ B activity might play in the reported biological activities of ESM.

Materials and methods

Reagents

All chemicals and reagents were used as received unless otherwise indicated. High-performance liquid chromatography (HPLC) grade water, sodium bicarbonate, sodium carbonate monohydrate, sodium hydroxide, and filter paper (P4 grade, medium-fine porosity) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Purified alkaline protease from *Bacillus licheniformis* was obtained from Bio-Cat, Inc. (Troy, VA, USA). ESM (from *Gallus gallus*) used in the preparation of ESM hydrolyzates was obtained from ESM Technologies, LLC (Carthage, MO, USA). Phosphate-buffered saline (PBS, pH 7.4), Roswell Park Memorial Institute-1640 culture medium, fetal bovine serum (FBS), L-glutamine 200 mM, penicillin–streptomycin 100X solution, glacial acetic acid, and methanol were obtained from Sigma-Aldrich Co (St Louis, MO, USA). TransAM[®] NF- κ B p65 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Active Motif (Carlsbad, CA, USA). Bradford method protein assay kits were obtained from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

Preparation of ESM hydrolyzates

Fermentation hydrolyzate

ESM powder (2.8 g) was suspended in 50 mL of purified water (HPLC grade) in a 100 mL round-bottomed flask, followed by the addition of 1.4 g of sucrose and 230 μ L of a proprietary inoculum of bacteria and yeasts. The slurry was allowed to ferment at room temperature (\sim 25°C) for 6 hours with moderate stirring (\sim 500 rpm). The slurry was vacuum filtered to remove unreacted solids. The resulting clear, light brown solution contains 5%–7% dissolved solids and was divided into \sim 1 mL aliquots, which were stored frozen (-20°C) until later use in the bioassays. This preparation is referred to as fermentation hydrolyzate of ESM (ESM-FH).

Enzymatic hydrolyzate

ESM powder (1.0 g) was suspended in a buffer mixture (pH 9.3) comprising 15 mL of 0.1 M sodium bicarbonate and 5 mL of 0.1 M sodium carbonate in a 50 mL round-bottomed flask. Alkaline protease powder (20% w/w to ESM) was added, and the slurry was allowed to stir (\sim 500 rpm) at 60°C for 5 days. The slurry was vacuum filtered to remove unreacted solids. The resulting clear, light brown solution contains 5%–7% dissolved solids and was divided into \sim 1 mL aliquots, which were stored frozen (-20°C) until later use in the bioassays. This preparation is referred to as enzymatic hydrolyzate of ESM (ESM-EH).

Chemical hydrolyzate

The hydrolysis was performed according to the method of Strohschein et al.³⁹ Briefly, ESM powder (1.0 g) was suspended in a 5% (w/v) sodium hydroxide solution in a 25 mL round-bottomed flask. The slurry was allowed to stir (~500 rpm) at 50°C for 4 hours. The mixture was allowed to cool and was vacuum filtered to remove any eggshell. The pH of the filtrate was adjusted to ~7 with a 5% (w/v) acetic acid solution while keeping the mixture temperature at $\leq 4^\circ\text{C}$. The solution was desalted using a dialyzing membrane with two to three equal volumes of water. The resulting clear, pale yellow solution was adjusted to contain 5%–7% dissolved solids and was divided into ~1 mL aliquots, which were stored frozen (-20°C) until later use in the bioassays. This preparation is referred to as chemical hydrolyzate of ESM (ESM-CH).

Cell source and culture conditions

Human peripheral blood mononuclear cells (PBMCs) were obtained from Astarte Biologics, LLC (Bothell, WA, USA). PBMCs were cultured at 3.2×10^6 cells/well in culture medium (RPMI-1640 medium supplemented with 10% FBS, L-glutamine [2 mM], penicillin [100 U/mL], and streptomycin [100 mg/mL]). The human leukemic monocyte cell line THP-1 was obtained from American Type Culture Collection (Manassas, VA, USA) and was cultured at 2.0×10^5 cells/well in culture medium (RPMI-1640 medium supplemented with 10% FBS, 2-mercaptoethanol [0.05 mM], and 1% penicillin–streptomycin). Both PBMC and THP-1 cell cultures were utilized for the subsequent determination of NF- κ B activity.

Evaluation of the in vitro NF- κ B activity of ESM hydrolyzates

The in vitro NF- κ B activity of ESM hydrolyzates was initially evaluated in human PBMCs utilizing the facilities and services of NIS Labs (Klamath Falls, OR, USA). PBMCs were plated at a density of 3.2×10^6 cells/well in culture medium in 12-well culture plates (growth area 3.8 cm²/well). The primary cells were plated in duplicate in two independent experiments ($n=2$ per condition). Aliquots of ESM hydrolyzates (ESM-FH or ESM-EH) were allowed to thaw and were diluted 1:1 with PBS prior to filtering through a 0.22 micron cellulose acetate disc to sterilize them. An additional two tenfold dilutions of the ESM hydrolyzates were prepared with culture medium. Each dilution (60 μL) was added to a culture well (3 mL total volume), resulting in final ESM hydrolyzate dilutions of 1:100, 1:1,000, and 1:10,000, and the cells were incubated at 37°C, 5% CO₂ for 4 hours. Not knowing whether ESM hydrolyzates

would be activating or inhibitory, a known activator of NF- κ B, lipopolysaccharide (LPS; 100 ng/mL), was used as a positive control, and a known inhibitor of NF- κ B, vitamin C (ascorbic acid; 20 mM),⁴⁰ was used as a comparator of the inhibitory effect on LPS-treated cells. Activity was also compared with untreated cells. Cells were pelleted by centrifuging at 150 x g (1,200 rpm) for 3 minutes and the supernatant was discarded. Cell lysates were prepared from the incubated cultures via bead milling. The whole cell lysates were assayed for NF- κ B activity via a commercially available ELISA kit according to the manufacturer's instructions. Samples were assayed in duplicate and were normalized for total protein content via the Bradford method according to the manufacturer's instructions.

The determination of the in vitro NF- κ B activity of ESM hydrolyzates in THP-1 cells was performed utilizing the facilities and services of the Jordan Valley Innovation Center within the Center for Biomedical and Life Sciences at Missouri State University (JVIC-MSU; Springfield, MO, USA). THP-1 cells were incubated in suspension for 3–5 days and were subsequently plated at a cell density of 2.0×10^5 cells/well in culture medium in 24-well culture plates (growth area 1.9 cm²/well). The cells were plated using a volume of 350 μL per well in duplicate in three independent experiments ($n=3$ per condition). Aliquots of ESM hydrolyzates (ESM-FH, ESM-EH, or ESM-CH) were allowed to thaw prior to filtering through a 0.2 micron polyethersulfone disc to sterilize them. The ESM hydrolyzates were then diluted (1:1,000; 1:10,000) with culture medium. Each dilution was added to a culture well (3 mL total volume), and the cells were incubated at 37°C, 5% CO₂ for 4 hours. Because ESM hydrolyzates were found to be activating in PBMCs, only LPS (10 ng/mL) was used as a positive control. Activity was again compared with untreated cells. Cells were pelleted by centrifuging at 150 x g (1,200 rpm) for 3 minutes and the supernatant was discarded. Cell lysates were prepared from the incubated cultures by adding 45 μL of complete lysis buffer (containing dithiothreitol and protease inhibitor). The whole cell lysates were assayed for NF- κ B activity via a commercially available ELISA kit according to the manufacturer's instructions. Samples were assayed in duplicate and were normalized for total protein content via the Bradford method according to the manufacturer's instructions.

Evaluation of the ESM source of NF- κ B activation

An initial evaluation of the source of NF- κ B activity was performed utilizing the facilities of NIS Labs using respective appropriate controls. PBMCs (prepared as described

previously) were exposed to aliquots of ESM hydrolyzate or control solutions for 4 hours prior to NF- κ B activity determination. ESM-FH NF- κ B activity was compared with untreated cells and cells treated with the fermentation broth (minus ESM). Similarly, the ESM-EH was compared with untreated cells and cells treated with the enzyme digest solution alone. A further, more discerning, evaluation of the source of in vitro NF- κ B activity of ESM hydrolyzates in THP-1 cells was performed utilizing the facilities and services of JVIC-MSU. THP-1 cells were prepared as described previously. Aliquots of ESM hydrolyzates (ESM-FH, ESM-EH, or ESM-CH) were allowed to thaw prior to filtering through a 0.2 micron polyethersulfone disc to sterilize them. The ESM hydrolyzates were then diluted (1:1,000; 1:10,000) with culture medium. Each dilution was added to a culture well as well as separate wells for hydrolyzate + polymyxin B (10 mg/mL) and hydrolyzate + lipoprotein lipase (10 mg/mL). The cells were incubated at 37°C, 5% CO₂ for 4 hours. LPS (10 ng/mL) was again used as a positive control. Activity was compared with untreated cells, cells treated with polymyxin B only, cells treated with LPS + polymyxin B, and cells treated with lipoprotein lipase only. Cell cultures were processed and whole cell lysates were analyzed as described previously.

Statistical analysis

Statistical analyses were done either as a two-tailed, independent group Student's *t*-test or a Kruskal–Wallis test for

multiple comparisons. Statistical significance was accepted at a *P*-value of *P*<0.05. SYSTAT software (version 13) was used for all statistical analyses.⁴¹

Results

Evaluation of the in vitro NF- κ B activity of ESM hydrolyzates

There were statistically significant levels of NF- κ B activation versus untreated cells at all three dilutions (1:100, 1:1,000, and 1:10,000) for the ESM-FH (*P*=0.021, *P*=0.020, *P*=0.009, respectively) in PBMCs. The ESM-EH also showed statistically significant levels of activation at the 1:100 and 1:1,000 dilutions (*P*=0.004, *P*=0.006, respectively) but fell just shy of significance at the 1:10,000 dilution (*P*=0.073). Both the fermentation and enzymatic hydrolyzates of ESM at the 1:100 and 1:1,000 dilutions activated NF- κ B in human PBMCs comparably with LPS at 100 ng/mL (Figure 1).

Similarly, there were statistically significant levels of NF- κ B activation versus untreated cells at both dilutions (1:1,000 and 1:10,000) for the fermentation and enzymatic hydrolyzates of ESM (ESM-FH: *P*=0.021, *P*=0.002; ESM-EH: *P*=0.007, *P*=0.007, respectively) in THP-1 cells. The ESM-CH also caused statistically significant levels of activation at the 1:1,000 dilution (*P*=0.005) but failed to differ from untreated cells at the 1:10,000 dilution (*P*=0.193). Both the fermentation and enzymatic hydrolyzates of ESM at the

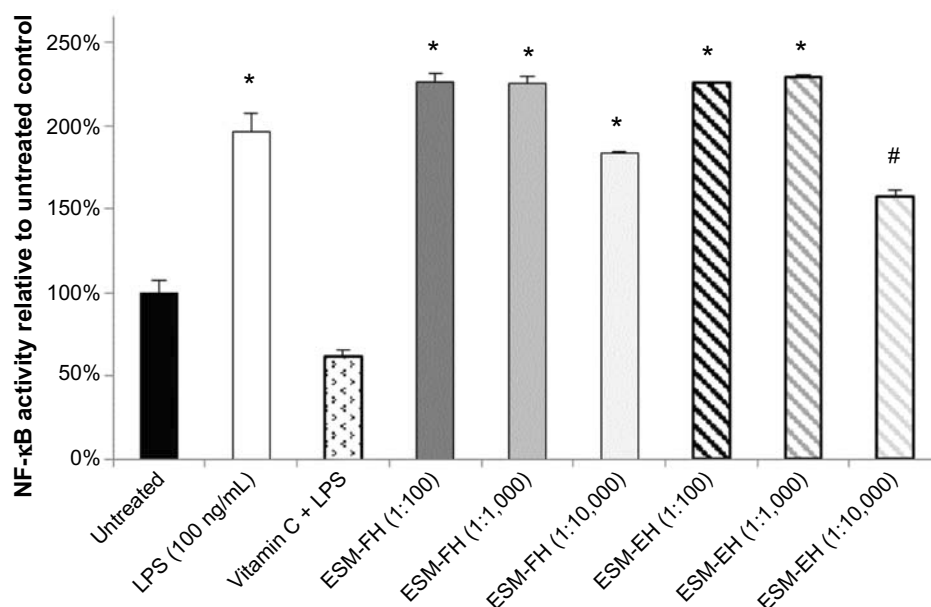


Figure 1 NF- κ B activity of fermentation and enzymatic hydrolyzates of ESM in human PBMCs. Human PBMCs were incubated for 4 hours with either medium (untreated control), LPS, vitamin C + LPS, or each dilution of ESM hydrolyzate. Whole cell lysates were then analyzed for NF- κ B activity after normalizing for protein content. Bars represent mean \pm standard error of the mean; values are presented relative to untreated control.

Notes: **P*<0.05 versus untreated control; #*P*<0.10 versus untreated control.

Abbreviations: NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B-cells; ESM, egg shell membrane; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; ESM-FH, ESM fermentation hydrolyzate; ESM-EH, ESM enzymatic hydrolyzate.

1:1,000 dilution activated NF- κ B in human THP-1 cells comparably with LPS at 10 ng/mL (Figure 2).

Evaluation of the ESM source of NF- κ B activation

No increased NF- κ B activity was observed in the cells treated with fermentation broth alone; however, ESM-FH activity was similar to what was observed previously (data not shown). Similarly, no increased NF- κ B activity was observed with enzyme digest alone; however, ESM-EH activity was similar to what was observed previously (data not shown). Polymyxin B (10 mg/mL) adequately inhibited activation by LPS at 10 ng/mL (Figure 3) but showed a small degree of NF- κ B activation itself (although not statistically significant). Lipoprotein lipase had no significant impact on NF- κ B activity when used alone. These effects were confirmed in the separate bioassays of all three hydrolyzates (data not shown). There was no statistically significant difference between the ESM hydrolyzate alone samples and the ESM hydrolyzate + polymyxin B samples for any of the hydrolyzates tested (1:10,000 dilution) (ESM-FH, $P=0.262$; ESM-EH, $P=0.727$; ESM-CH, $P=0.240$). Similarly, there was no statistically significant difference between the ESM hydrolyzate alone samples and the ESM hydrolyzate + lipoprotein lipase samples for any of the hydrolyzates tested (1:10,000 dilution) (ESM-FH, $P=0.539$; ESM-EH, $P=0.855$; ESM-CH, $P=0.281$). Data for ESM-FH are presented in Figure 4.

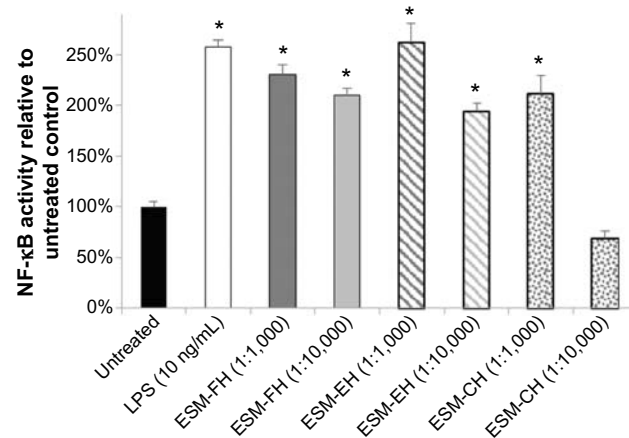


Figure 2 NF- κ B activity of fermentation, enzymatic, and chemical hydrolyzates of ESM in the human THP-1 monocyte cell line. Human THP-1 monocytes were incubated for 4 hours with either medium (untreated control), LPS, or each dilution of ESM hydrolyzate. Whole cell lysates were then analyzed for NF- κ B activity after normalizing for protein content. Bars represent mean \pm standard error of the mean; values are presented relative to untreated control.

Note: * $P < 0.05$ versus untreated control.

Abbreviations: NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B-cells; ESM, egg shell membrane; LPS, lipopolysaccharide; ESM-FH, ESM fermentation hydrolyzate; ESM-EH, ESM enzymatic hydrolyzate; ESM-CH, ESM chemical hydrolyzate.

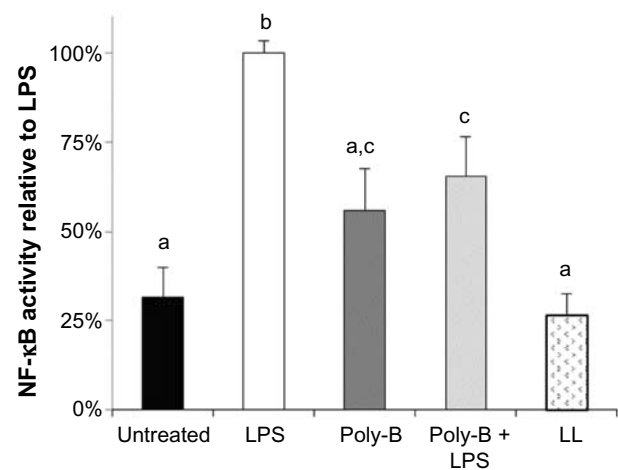


Figure 3 Effects of poly-B and LL on in vitro NF- κ B activity in untreated and LPS-treated THP-1 monocytes. Human THP-1 monocytes were incubated for 4 hours with either medium (untreated control), LPS, poly-B, poly-B + LPS, or LL. Whole cell lysates were then analyzed for NF- κ B activity after normalizing for protein content. Bars represent mean \pm standard error of the mean; values are presented relative to LPS.

Note: Columns with differing letters are statistically ($P < 0.05$) different.

Abbreviations: poly-B, polymyxin B; LL, lipoprotein lipase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B-cells; LPS, lipopolysaccharide.

Similar results were observed for ESM-EH and ESM-CH (data not shown).

Discussion

NF- κ B is of fundamental importance to the functioning of innate immunity. Its dysregulation is implicated in the pathology of a considerable variety of human diseases. The

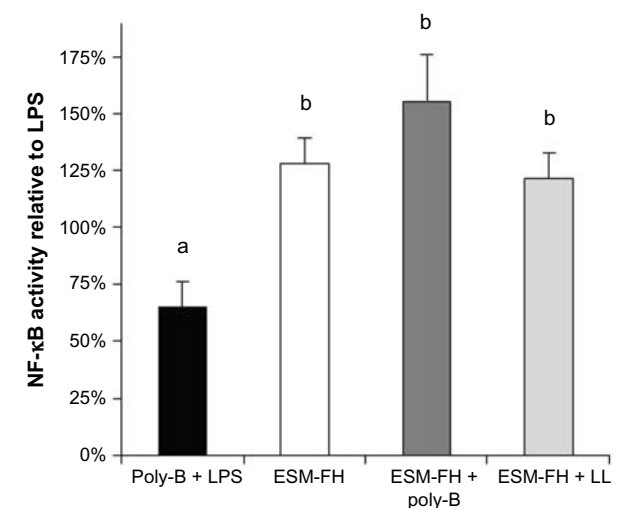


Figure 4 NF- κ B activity of a fermentation hydrolyzate of ESM in THP-1 monocytes with and without poly-B or LL. Human THP-1 monocytes were incubated for 4 hours with either poly-B + LPS, ESM-FH alone (1:10,000 dilution), ESM-FH + poly-B, or ESM-FH + LL. Whole cell lysates were then analyzed for NF- κ B activity after normalizing for protein content. Bars represent mean \pm standard error of the mean; values are presented relative to LPS.

Note: Columns with differing letters are statistically ($P < 0.05$) different.

Abbreviations: NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B-cells; ESM, egg shell membrane; poly-B, polymyxin B; LL, lipoprotein lipase; LPS, lipopolysaccharide; ESM-FH, ESM fermentation hydrolyzate.

goal of our studies was to investigate the potential role NF- κ B might play in the beneficial biological activities reported previously for ESM. ESM hydrolyzates were chosen for our studies because of 1) the simplicity of their addition to cell cultures, 2) the variety of preparation methods available (eg, fermentation, enzymatic, or chemical), and 3) the potential to generate bioactive substances possessing differing activity profiles. Human PBMCs obtained from healthy volunteers were initially chosen for these studies owing to the wide variety of mononuclear cells present in this source (eg, T-lymphocytes, B-lymphocytes, natural killer cells, monocytes, and dendritic cells).^{42,43} Readily available THP-1 monocytes were subsequently used because of their antigen presenting characteristics that are similar to those of dendritic cells found in the gut, which are not readily available.

At the outset, it was unknown whether ESM hydrolyzates would stimulate or inhibit active levels of NF- κ B. Previous work⁴ in which an extract of ESM was shown to reduce the expression of proinflammatory cytokines in mitogen-challenged human PBMCs provided preliminary data that the hydrolyzates' effect on NF- κ B would be inhibitory. The data reported here show that ESM hydrolyzates significantly activate NF- κ B in unstimulated cells. The ESM-FH and the ESM-EH showed quite similar activities in both PBMCs and THP-1 cell cultures. The ESM-CH, however, was about ten-fold less active than the other hydrolyzates in THP-1 monocytes. As seen in Figure 2, the 1:1,000 dilution of ESM-CH had approximately the same activity as the 1:10,000 dilutions of ESM-FH and ESM-EH. This result is logically consistent with the differing specificities of the hydrolysis conditions. That is, both microorganisms and purified enzymes will be fairly specific in their sites of hydrolysis within the ESM protein structure, whereas chemical hydrolysis is quite indiscriminate. It is likely that the NF- κ B-activating soluble peptides that are formed initially during hydrolysis are being further degraded by the harsh chemical conditions, resulting in lower activity in cell culture.

Because ESM hydrolyzates induced increased levels of NF- κ B, we felt it necessary to broadly explore the possible sources of this activity. Our immediate concern was to determine whether the activity was inherent in ESM or if it was due to materials used to produce the hydrolyzates (ie, fermentation inoculum or enzyme) or to some other type of contaminant. As neither the fermentation inoculum nor the enzyme produced elevated NF- κ B activity, it was necessary to consider alternative sources for the observed activity. Degradation products of extracellular matrix components, such as fibronectin, collagen, proteoglycans, and hyaluronan, are

believed to play a significant role in the pathogenesis of both osteo- and rheumatoid arthritis, likely through autoimmune mechanisms,⁴⁴⁻⁴⁶ and at least some of these components do so via activation of NF- κ B.^{47,48} Hyaluronan,³ proteoglycans,² and collagens¹ all naturally occur in ESM and may be responsible for at least part of the activity reported here. However, they occur at relatively low levels, up to 2% each hyaluronan and proteoglycans and up to 5% collagen. Therefore, we speculate that other sources may provide a more likely explanation for the observed stimulatory activity.

Bacterial cellular components and DNA have been found in the joints of patients suffering from a variety of arthritides,⁴⁹ and the innate immune response to these contaminants is believed to play a possible role in the pathogenesis of these diseases.^{50,51} Additionally, Pugh et al⁵² found that the majority of in vitro macrophage activation exhibited by extracts of a number of common "immune enhancing" botanicals (Echinacea, ginseng, and alfalfa) was not inherent in the botanical compounds themselves but was due to contamination with bacterial cellular components such as bacterial lipoproteins and LPS. Being a natural product, it was logical that ESM would be contaminated with various microbial species. Although the ESM was heat treated to substantially reduce the living bioburden prior to hydrolysis, the cellular components from the formerly living microorganisms would remain.

Intact microorganisms should have been removed during filtration of the hydrolyzates through 0.2 micron filter membranes prior to the initial assays; however, cellular fragments from these microorganisms could not be ruled out as the source of activation. Therefore, we investigated the possible contribution of LPS and/or bacterial lipoprotein to the observed activation of NF- κ B by ESM hydrolyzates. LPS activity is abrogated by the long-known antibiotic polymyxin B,⁵³ and the enzyme lipoprotein lipase deactivates bacterial lipoprotein through cleavage of the lipid-protein bond.⁵² All three hydrolyzates (ESM-FH, ESM-EH, and ESM-CH) were evaluated alone and in combination with either polymyxin B or lipoprotein lipase. If a significant proportion of the NF- κ B activity of the hydrolyzates was derived from either of these bacterial cellular components, the polymyxin B-treated and lipoprotein lipase-treated cells would have exhibited substantially decreased activity compared with the hydrolyzates alone. However, no differences were observed between the ESM hydrolyzates alone and either the ESM hydrolyzates + polymyxin B or ESM hydrolyzates + lipoprotein lipase. With no evidence that the NF- κ B activation by ESM hydrolyzates is derived from bacterial cellular components, it would seem that this activity is attributable to the inherent bioactive components present

in ESM. Further work will be needed to elucidate whether this activity draws from known stimulatory components of ESM (hyaluronan, proteoglycans, collagens) or from an as yet unidentified peptide or peptides produced from the various hydrolysis conditions.

Since the vast majority of NF- κ B dysregulation encountered in disease pathology is that of elevated or inappropriately activated NF- κ B,^{24–27} it would seem counterintuitive that ESM hydrolyzates activating NF- κ B would contribute to ESM's observed in vivo efficacy. Here we raise the possibility that this efficacy is achieved through an “oral tolerance” mechanism. Oral tolerance refers to the phenomenon of a reduced peripheral immune response (tolerance) that results from the repeated exposure of the mucosal immune system in the gut to ingested protein antigens. Oral tolerance to immunogenic peptides that are repeatedly ingested is believed to result from immune surveillance within the gut-associated lymphoid tissue as a way for the body to prevent an inappropriate or unnecessary immune response to proteins normally consumed in the diet. This tolerogenic behavior is thought to occur through the presentation of these peptides by antigen-presenting cells in the gut-associated lymphoid tissue to circulating T-cells, causing them to transform into regulatory T-cells (T_{reg} cells), which results in anergy and even active suppression by these cells when they return to circulation.^{54,55} Oral tolerance has been shown to be effective in a variety of autoimmune diseases, including arthritis, diabetes, colitis, and multiple sclerosis (see⁵⁶ and⁵⁷ for a review). There has been particular attention paid to autoimmune arthritides in this area of research in which cartilage autoantigens (particularly type II collagen) produce antigen-specific suppression of the immune response. In addition, “bystander suppression”, wherein T_{reg} cells produce a higher proportion of general anti-inflammatory cytokines (eg, IL-4, IL-10, and transforming growth factor-beta) suppressing the local autoimmune reaction independently of the antigen type, is also an active area of arthritis research.^{58,59} Further work will be needed to investigate whether ESM might function via 1) antigen-specific immune suppression from its extracellular matrix content (ie, collagen, proteoglycans, and hyaluronan), 2) bystander suppression, 3) some unrelated mechanism, or 4) some combination of these.

Conclusion

A great deal of research has been conducted relating to NF- κ B since its discovery, particularly in the last couple of decades, and with so many people suffering from diseases involving its dysregulation, this trend is sure to increase. We presented here results that ESM hydrolyzates produced

via three different hydrolysis conditions (ie, fermentation, enzymatic, and chemical) activate NF- κ B in both human primary cells and a human leukemic cell line. The source of this activity was also investigated to confirm that it is inherent to ESM and not derived from hydrolysis conditions or from bacterial contamination. We also proposed a plausible hypothesis, via an oral tolerance mechanism, as to how this NF- κ B activity might translate into the in vivo efficacy that has been previously observed with ESM. Further work is needed to confirm this proposed mechanism of action and to possibly identify the component(s) of ESM that is/are the source of the reported activity.

Acknowledgment

The sponsor of the studies was ESM Technologies, LLC.

Author contributions

KJR conceived of the study and participated in its design and coordination, conducted the statistical analyses, and drafted the manuscript. PLD participated in the design of the study and helped to draft the manuscript. AO participated in the design of the study, carried out the cell culture work and immunoassays, and reviewed the manuscript. FDL participated in the design of the study, prepared all of the hydrolyzates for use in the study, and reviewed the manuscript. All authors read and approved the final manuscript.

Disclosure

KJR and FDL are employed by the sponsor of the studies. PLD and AO have no competing interests.

References

1. Wong M, Hendrix MJC, von der Mark K, Little C, Stern R. Collagen in the egg shell membranes of the hen. *Dev Biol*. 1984;104(1):28–36.
2. Baker JR, Balch DA. A study of the organic material of hen's-egg shell. *Biochem J*. 1962;82:352–361.
3. Long FD, Adams RG, DeVore DP, inventors. Preparation of hyaluronic acid from eggshell membrane. United States patent US 6946551. September 20, 2005.
4. Benson KF, Ruff KJ, Jensen GS. Effects of natural eggshell membrane (NEM) on cytokine production in cultures of peripheral blood mononuclear cells: increased suppression of tumor necrosis factor- α levels after in vitro digestion. *J Med Food*. 2012;15(4):360–368.
5. Ruff KJ, DeVore DP. Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Mod Res Inflamm*. 2014;3(1):19–25.
6. Ruff KJ, DeVore DP, Leu MD, Robinson MA. Eggshell membrane: a possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin Interv Aging*. 2009;4:235–240.
7. Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW. Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin Rheumatol*. 2009;28:907–914.

8. Danesch U, Seybold M, Rittinghausen R, Treibel W, Bitterlich N. NEM® brand eggshell membrane effective in the treatment of pain associated with knee and hip osteoarthritis: results from a six-center, open-label German clinical study. *J Arthritis*. 2014;3(3):136.
9. Bauer KL, Dierenfeld ES, Hartup BK. Evaluation of a nutraceutical joint supplement in cranes. *Proc North Am Crane Workshop*. 2014;12: 27–32.
10. Dierenfeld ES, Baum D, Hampe L, Jensen J, Atwell C, Wedekind K. Evaluation of a nutraceutical joint supplement in camels. *Amer Hol Vet Med Assoc J*. 2014;39:59–66.
11. Jia H, Saito K, Aw W, et al. Transcriptional profiling in rats and an ex vivo analysis implicate novel beneficial function of egg shell membrane in liver fibrosis. *J Funct Foods*. 2013;5:1611–1619.
12. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*. 1986;46:705–716.
13. Gilmore TD. Introduction to NF- κ B: players, pathways, perspectives. *Oncogene*. 2006;25:6680–6684.
14. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124:783–801.
15. Hayden M, West A, Ghosh S. NF- κ B and the immune response. *Oncogene*. 2006;25:6758–6780.
16. Beg A, Finco T, Nantermet P, Baldwin A. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I κ B α : a mechanism for NF- κ B activation. *Mol Cell Biol*. 1993;13(6):3301–3310.
17. Flodström M, Welsh N, Eizirik D. Cytokines activate the nuclear factor κ B (NF- κ B) and induce nitric oxide production in human pancreatic islets. *FEBS Letters*. 1996;385:4–6.
18. Schreck R, Albermann K, Baeuerle P. Nuclear factor κ B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Rad Res Comm*. 1992;17(4):221–237.
19. Scandalios J. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Braz J Med Biol Res*. 2005;38:995–1014.
20. Cooper S, Bowden G. Ultraviolet B regulation of transcription factor families: roles of nuclear factor-kappa B (NF- κ B) and activator protein-1 (AP-1) in UVB-induced skin carcinogenesis. *Curr Cancer Drug Targets*. 2007;7:325–334.
21. Reelfs O, Tyrrell R, Pourzand C. Ultraviolet A radiation-induced immediate iron release is a key modulator of the activation of NF- κ B in human skin fibroblasts. *J Invest Dermatol*. 2004;122:1440–1447.
22. Kono H, Rusyn I, Yin M, et al. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *J Clin Invest*. 2000;106:867–872.
23. She H, Xiong S, Lin M, Zandi E, Giulivi C, Tsukamoto H. Iron activates NF- κ B in Kupffer cells. *Am J Physiol Gastrointest Liver Physiol*. 2002;283:G719–G726.
24. Yamamoto Y, Gaynor R. Role of the NF- κ B pathway in the pathogenesis of human disease states. *Curr Mol Med*. 2001;1:287–296.
25. Prajapati B, Singhal M, Yashwant, Sharma G, Gupta V. Role of NF κ B in various immunological and inflammatory disorders. *Int J Toxicol Pharmacol Res*. 2010;2(1):35–39.
26. Courtis G, Gilmore T. Mutations in the NF- κ B signaling pathway: implications for human disease. *Oncogene*. 2006;25:6831–6843.
27. Tak P, Firestein G. NF- κ B: a key role in inflammatory diseases. *J Clin Invest*. 2001;107(1):7–11.
28. Brand K, Page S, Rogler G, et al. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest*. 1996;97(7):1715–1722.
29. Hajra L, Evans A, Chen M, Hyduk S, Collins T, Cybulsky M. The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc Natl Acad Sci U S A*. 2000;97(16):9052–9057.
30. Akama K, Albanese C, Pestell R, Van Eldik L. Amyloid β -peptide stimulates nitric oxide production in astrocytes through an NF κ B-dependent mechanism. *Proc Natl Acad Sci U S A*. 1998;95(10): 5795–5800.
31. Akama K, Van Eldik L. β -amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1 β - and tumor necrosis factor- α (TNF α)-dependent, and involves a TNF α receptor-associated factor- and NF κ B-inducing kinase-dependent signaling mechanism. *J Biol Chem*. 2000;275(11):7918–7924.
32. Meterski B, Bohringer S, Klein W, et al. Inhibitors in the NF κ B cascade comprise prime candidate genes predisposing to multiple sclerosis, especially in selected combinations. *Genes Immun*. 2002;3: 211–219.
33. Yan J, Greer J. NF-kappa B, a potential therapeutic target for the treatment of multiple sclerosis. *CNS Neurolog Disord Drug Targets*. 2008;7(6):536–557.
34. Patel S, Santani D. Role of NF- κ B in the pathogenesis of diabetes and its associated complications. *Pharmacol Rep*. 2009;61:595–603.
35. Andreassen A, Kelly M, Berg R, Møller K, Pedersen B. Type 2 diabetes is associated with altered NF- κ B DNA binding activity, JNK phosphorylation, and AMPK phosphorylation in skeletal muscle after LPS. *PLoS One*. 2011;6(9):e23999.
36. Basseres D, Baldwin A. Nuclear factor- κ B and inhibitor of κ B kinase pathways in oncogenic initiation and progression. *Oncogene*. 2006;25: 6817–6830.
37. Kordes U, Krappmann D, Heissmeyer V, Ludwig W, Scheidereit C. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. *Leukemia*. 2000;14:399–402.
38. Bauerle KT, Schweppe RE, Haugen BR. Inhibition of nuclear factor-kappa B differentially affects thyroid cancer cell growth, apoptosis, and invasion. *Mol Cancer*. 2010;9:117.
39. Stroehbehn RE, Etzel LR, Figgins J, inventors. Solubilized protein composition obtained from avian eggshell membrane. United States patent US 8173174. May 8, 2012.
40. Bowie AG, O'Neill LAJ. Vitamin C inhibits NF- κ B activation by TNF via the activation of p38 mitogen-activated protein kinase. *J Immunol*. 2000;165(12):7180–7188.
41. Systat Software, Inc. Available at: <http://www.systat.com>. Accessed January 6, 2015.
42. Reichert T, DeBruyere M, Deneys V, et al. Lymphocyte subset reference ranges in adult caucasians. *Clin Immunol Immunopathol*. 1991;60: 190–208.
43. Jentsch-Ullrich K, Koenigsmann M, Mohren M, Franke A. Lymphocyte subsets' reference ranges in an age- and gender-balanced population of 100 healthy adults: a monocentric German study. *Clin Immunol*. 2005;116(2):192–197.
44. Homandberg GA, Davis G, Maniglia C, Shrikhande A. Cartilage chondrolysis by fibronectin fragments causes cleavage of aggrecan at the same site as found in osteoarthritic cartilage. *Osteoarthritis Cartilage*. 1997;5(6):450–453.
45. Yoshida M, Tsuji M, Kurosaka D, et al. Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. *Mod Rheumatol*. 2006;16(5): 276–281.
46. György B, Tóthfalusi L, Nagy G, et al. Natural autoantibodies reactive with glycosaminoglycans in rheumatoid arthritis. *Arthritis Res Ther*. 2008;10:R110.
47. Noble PW, McKee CM, Cowman M, Shin HS. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. *J Exp Med*. 1996;183(5):2373–2378.
48. Saklatvala J. Inflammatory signaling in cartilage: MAPK and NF- κ B pathways in chondrocytes and the use of inhibitors for research into pathogenesis and therapy of osteoarthritis. *Curr Drug Targets*. 2007;8: 305–313.
49. Van Der Heijden IM, Wilbrink B, Tchetverikov I, et al. Presence of bacterial DNA and bacterial peptidoglycans in joints of patients with rheumatoid arthritis and other arthritides. *Arthritis Rheum*. 2000;43(3): 593–598.
50. Scanzello CR, Plaas A, Crow MK. Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound? *Curr Opin Rheumatol*. 2008;20(5):565–572.

51. Klinman D. Does activation of the innate immune system contribute to the development of rheumatoid arthritis? *Arthritis Rheum.* 2003;48(3): 590–593.
52. Pugh ND, Tamta H, Balachandran P, et al. The majority of in vitro macrophage activation exhibited by extracts of some immune enhancing botanicals is due to bacterial lipoproteins and lipopolysaccharides. *Int Immunopharmacol.* 2008;8(7):1023–1032.
53. Morrison DC, Jacobs DM. Inhibition of lipopolysaccharide-initiated activation of serum complement by polymyxin B. *Infect Immun.* 1976;13(1):298–301.
54. Friedman A, Weiner HL. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci U S A.* 1994;91(14):6688–6692.
55. Faria AMC, Weiner HL. Oral tolerance. *Immunol Rev.* 2005;206(1): 232–259.
56. Garside P, Mowat AM, Khoruts A. Oral tolerance in disease. *Gut.* 1999;44(1):137–142.
57. Faria AMC, Weiner HL. Oral tolerance: therapeutic implications for autoimmune diseases. *Clin Dev Immunol.* 2006;13(2–4):143–157.
58. Stancikova M, Stancik R, Gubzova Z, Rovensky J. Collagen in the treatment of rheumatic diseases: oral tolerance. *Bratisl Med J.* 1999;100(10):567–571.
59. Broere F, Wieten L, Koerkamp EIK, et al. Oral or nasal antigen induces regulatory T cells that suppress arthritis and proliferation of arthritogenic T cells in joint draining lymph nodes. *J Immunol.* 2008;181(2): 899–906.

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NEM® Brand Eggshell Membrane Effective in the Treatment of Pain Associated with Knee and Hip Osteoarthritis: Results from a Six Center, Open Label German Clinical Study

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Abstract

Objective: NEM® brand eggshell membrane is a novel dietary supplement ingredient that contains naturally occurring glycosaminoglycans and proteins essential for maintaining healthy joints. A six center, open label clinical study was conducted to evaluate the efficacy and safety of NEM® as a treatment for pain and inflexibility associated with osteoarthritis of the knee and/or hip in a European population.

Methods: Forty-four subjects received oral NEM® 500 mg once daily for eight weeks. The primary outcome measure was to evaluate the mean effectiveness of NEM® in relieving general pain associated with moderate osteoarthritis of the knee and/or hip at 10, 30 and 60 days utilizing a 10-question abbreviated questionnaire based on the WOMAC osteoarthritis questionnaire.

Results: Supplementation with NEM® produced a significant treatment response from baseline at 10 days (Q1-6 and Q9) (8.6% to 18.1% improvement) and at 30 and 60 days for all nine pain-related questions evaluated (22.4% to 35.6% improvement) and at 30 and 60 days for stiffness (Q10) (27.4% to 29.3% improvement). In a Patient's Global Assessment, greater than 59% of patients rated the efficacy of NEM® as good or very good following 60 days of supplementation. Physicians also rated the treatment effective in subjects, with greater than 75% having moderate or significant improvement from baseline after 60 days. There were no serious adverse events reported during the study and the treatment was reported to be well tolerated.

Conclusions: Supplementation with NEM® significantly reduced pain, both rapidly (10 days) and continuously (60 days) demonstrating that it is a safe and effective therapeutic option for the treatment of pain associated with osteoarthritis of the knee and/or hip. Results from previous clinical studies on NEM® can likely be extended to the broader European population.

Keywords: Knee, Hip, Osteoarthritis, Eggshell membrane, NEM, Dietary supplement, Glycosaminoglycans

Introduction

Estimates of the prevalence of osteoarthritis (OA) in European populations vary widely, however a recent study [1] from a region in Spain places the prevalence of knee OA at 12.2% and that of hip OA at 7.4%. The pain associated with these maladies can be quite debilitating and few treatment options exist outside of easing symptoms. This usually involves the use of analgesics (i.e. acetaminophen, oxycodone, propoxyphene) or non-steroidal anti-inflammatory drugs (NSAIDs) (i.e. ibuprofen, diclofenac, celecoxib), alone or in combination. Most of these treatments have shown limited effectiveness in randomized controlled clinical trials (RCTs) [2-5] or are known to have significant and sometimes severe side effects. NEM® brand eggshell membrane has previously demonstrated good efficacy in relieving pain and stiffness associated with OA of the knee in an RCT [6] and has shown similar efficacy in limited trials for other affected joints [7].

Eggshell membrane is primarily composed of fibrous proteins such as Collagen Type I [8]. However, eggshell membranes have also been shown to contain other bioactive components, namely glycosaminoglycans (i.e. dermatan sulfate, chondroitin sulfate and hyaluronic acid and keratan sulfate) [9-11]. A number of these constituents have been shown previously to be beneficial in the treatment of OA [12,13]. Eggshell membrane itself has been shown both *in vitro* [14] and *in vivo* [15] to reduce various pro-inflammatory

cytokines, including interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α), two primary mediators of inflammation. A U.S. company, ESM Technologies, LLC (Carthage, MO USA), has developed methods to efficiently and effectively separate eggshell membrane from eggshells on a commercial metric-ton scale. The isolated membrane is then partially hydrolyzed using a proprietary process and dry-blended to produce NEM® brand eggshell membrane. Compositional analysis of NEM® conducted by the manufacturer has identified a high content of protein and moderate quantities of glucosamine (up to 1% by dry weight), chondroitin sulfate (up to 1%), hyaluronic acid (up to 2%), and collagen (Type I, up to 5%).

The multi-center trial reported herein was designed to evaluate the acceptability of this natural arthritis treatment with European orthopedic surgeons and patients. Success of this trial would also

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validate the extension of the body of clinical evidence for NEM® from the United States to a European population. Therefore, a 2-month open-label study was conducted at six different clinical sites throughout Germany to evaluate the efficacy and tolerability of NEM® for the relief of the pain and discomfort associated with osteoarthritis of the knee and/or hip.

Materials and Methods

Study design

The study was conducted according to a prospective, multi-center, open label design and was conducted in Germany in accordance with the International Conference on Harmonization guideline for the principles of Good Clinical Practice (ICH E6) and the Declaration of Helsinki to ensure protection of human subjects. Patients provided their written informed consent to participate. Neither the clinical investigators nor the patients were blinded to treatment (open label design). Treatment consisted once daily orally of Atrosia® (Weber and Weber, GmbH and Co. KG, Germany) providing 500 mg of NEM® in vegetarian capsules that were stored in closed containers at ambient temperature. Clinic visits were scheduled for subjects at study initiation and at 60 days following the onset of treatment. Treatment compliance was checked at clinic visits by patient interview and by counting the number of unused doses of the study medications. Analgesics (i.e. acetaminophen) were allowed for rescue pain relief. However, subjects recorded the time and amount of analgesic taken in patient diaries so that overall analgesic use could be evaluated as part of the study.

Patients

All subjects 18 years of age or older who were seeking relief of mild to moderate pain due to osteoarthritis of the knee and/or hip were considered for enrollment in the study. In order to be eligible, subjects must have had moderate persistent pain in the knee and/or hip associated with osteoarthritis and must have had baseline scores within the range of 4-7 on the first three questions dealing with joint pain. Subjects that were currently taking analgesic medications or NSAIDs every day, currently taking glucosamine, chondroitin sulfate, MSM, or collagen were ineligible to participate in the study. Patients were excluded if they were currently receiving remission-inducing drugs such as methotrexate or immunosuppressive medications or had received them within the past 3 months. Other exclusionary criteria were: a known allergy to eggs or egg products, or pregnant or breastfeeding women. Subjects participating in any other research study involving an investigational product (drug, device, or biologic) or a new application of an approved product, within 30 days of screening were also excluded from participating in the trials.

Treatment response

The primary outcome measure of this study was to evaluate the mean effectiveness of NEM® in relieving general pain associated with moderate osteoarthritis of the knee and/or hip (Questions 1-9). Additional outcome measures were to evaluate general stiffness (Question 10) and analgesic use during the study. The primary treatment response endpoints were the 10-, 30-, and 60-day patient assessments utilizing a 10-question 'Short Form' questionnaire derived from the Western Ontario and McMaster Universities Osteoarthritis Index questionnaire (WOMAC), which has some precedence [16,17]. Each question included a zero to 10 analog Likert-scale, with zero equating to no pain (or no stiffness) and 10 equating to most severe pain (or most severe stiffness). Patients were asked to mark a number corresponding to the perceived pain (or stiffness) from the affected

treatment joint(s). Endpoints were then compared to pretreatment assessments. At the conclusion of the study, subjects were asked to provide a Patient's Global Assessment of treatment efficacy (4 categories-very good/good/moderate/poor) and tolerability (same 4 categories). Clinical investigators were also asked to provide a Physician's Global Assessment of treatment efficacy (5 categories-symptom-free/significant improvement/moderate improvement/unchanged/impaired).

Adverse events

A secondary objective of this study was to evaluate tolerability and any adverse reactions associated with supplementation with NEM®. The subject's self-assessment diaries were reviewed and any discomfort or other adverse events were recorded and reported in accordance with applicable ICH Guidelines. Adverse events and serious adverse events were assessed by the clinical investigator at each study visit and followed until resolution, as necessary. Serious adverse events were required to be reported to the clinical monitor immediately.

Statistical analysis

As this was an open-label study, a simple single group sample size estimate [18] was performed for statistical power determination for a continuous variable. In previous trials with NEM® [6,7], the standard deviation for the study subjects for pain (within the inclusion range of this study) averaged 34.6%. We hoped to be able to detect a 1.5 point difference from baseline within the 10-point Likert scale. Thus a minimum of 43 subjects would need to be enrolled to have a 95% likelihood of detecting the expected improvement with a statistical power of 80%. Comparisons of demographic data from the six clinical sites were made with a Kruskal-Wallis test for multiple independent samples at baseline. Statistical significance was accepted at $p < 0.05$. Post-baseline statistical analyses were done as repeated measures Analysis of Variance (rm-ANOVA) with a Greenhouse-Geisser correction. Items found to have statistical significance with rm-ANOVA were then compared using a Wilcoxon test for dependent samples. Statistical significance was accepted at $p < 0.05$. Analysis of the primary outcome measure (the change from baseline in general pain levels) was conducted in the per protocol population. SPSS Statistics V19.0 was used for all statistical analyses [19].

Results

Patient recruitment began in March 2012 at six clinical sites in Germany and the final follow-up was conducted in July 2012. A total of forty-four subjects between the ages of 32 and 95 were enrolled with

Age, yrs	67.1 ± 14.0
Sex	
Male (%)	17 (39)
Female (%)	27 (61)
Height, cm	170.2 ± 9.5
Weight, kg	74.2 ± 13.1
Body-mass Index	25.5 ± 4.1
Affected Joint	
Knee (l,r,bilateral)	39 (28,27,16)
Hip (l,r,bilateral)	14 (11,10,7)
Ankle (l,r,bilateral)	3 (2,2,1)

*Except where indicated otherwise, values are reported as mean ± standard deviation (SD) (n=44). BMI was determined as weight in kilograms divided by height in meters squared.

Table 1: Patient Demographics*.

Question 1: Pain when walking on level ground?	4.8 ± 1.0
Question 2: Pain when going up or down stairs?	5.7 ± 1.0
Question 3: Pain when at rest (i.e. sitting, lying down, etc.)?	5.3 ± 1.0
Question 4: Pain when sitting with legs bent for an extended period of time (i.e. in a car, at a theater, etc.)?	3.4 ± 1.8
Question 5: Pain when getting up from a seated position?	5.3 ± 1.3
Question 6: Pain when getting in and out of a car, a bathtub, etc.?	5.3 ± 1.1
Question 7: Pain when bending, stooping, or kneeling?	5.7 ± 1.3
Question 8: Pain when putting on socks or pantyhose?	4.4 ± 1.9
Question 9: Pain with light household chores (i.e. laundry, dusting, vacuuming, etc.)?	4.6 ± 1.7
Question 10: Stiffness when first getting up from bed in the morning?	4.2 ± 1.8

*Values are reported as mean ± standard deviation (SD) (n=37)

Table 2: Pooled baseline clinical characteristics for the 10-question patient questionnaire.

	Days Post-treatment	Mean ± SD	Percent Improvement	P-value [†]		Days Post-treatment	Mean ± SD	Percent Improvement	P-value [†]
Question 1	Baseline (n=37)	4.8 ± 1.0	-	-	Question 6	Baseline (n=37)	5.3 ± 1.1	-	-
	10 (n=37)	3.9 ± 1.7	18.1%	0.001*		10 (n=37)	4.4 ± 1.3	15.4%	0.001*
	30 (n=37)	3.3 ± 1.5	30.7%	<0.001*		30 (n=37)	3.7 ± 1.3	29.1%	<0.001*
	60 (n=37)	3.3 ± 1.8	32.4%	<0.001*		60 (n=37)	3.5 ± 1.6	32.8%	<0.001*
Question 2	Baseline (n=37)	5.7 ± 1.0	-	-	Question 7	Baseline (n=37)	5.7 ± 1.3	-	-
	10 (n=37)	4.7 ± 1.7	17.7%	0.001*		10 (n=37)	5.2 ± 1.7	8.6%	0.056
	30 (n=37)	4.1 ± 1.6	26.7%	<0.001*		30 (n=37)	4.4 ± 1.6	22.4%	<0.001*
	60 (n=37)	3.8 ± 1.8	32.6%	<0.001*		60 (n=37)	4.1 ± 1.7	28.0%	<0.001*
Question 3	Baseline (n=37)	5.3 ± 1.0	-	-	Question 8	Baseline (n=37)	4.4 ± 1.9	-	-
	10 (n=37)	4.5 ± 1.5	14.3%	0.001*		10 (n=37)	4.0 ± 1.7	9.2%	0.064
	30 (n=37)	3.8 ± 1.4	27.5%	<0.001*		30 (n=37)	3.2 ± 1.7	25.5%	<0.001*
	60 (n=37)	3.6 ± 1.5	32.6%	<0.001*		60 (n=37)	2.9 ± 1.7	33.6%	<0.001*
Question 4	Baseline (n=37)	3.4 ± 1.8	-	-	Question 9	Baseline (n=37)	4.6 ± 1.7	-	-
	10 (n=37)	2.9 ± 1.9	15.7%	0.042*		10 (n=37)	4.1 ± 1.4	11.7%	0.041*
	30 (n=37)	2.3 ± 1.4	33.8%	<0.001*		30 (n=37)	3.6 ± 1.4	23.0%	0.002*
	60 (n=37)	2.2 ± 2.0	35.6%	<0.001*		60 (n=37)	3.0 ± 1.7	34.9%	<0.001*
Question 5	Baseline (n=37)	5.3 ± 1.3	-	-	Question 10	Baseline (n=37)	4.2 ± 1.8	-	-
	10 (n=37)	4.7 ± 1.6	11.7%	0.012*		10 (n=37)	3.8 ± 1.9	9.9%	0.075
	30 (n=37)	4.0 ± 1.3	24.0%	<0.001*		30 (n=37)	3.0 ± 1.8	27.4%	<0.001*
	60 (n=37)	3.6 ± 2.0	31.7%	<0.001*		60 (n=37)	2.9 ± 1.7	29.3%	<0.001*

[†]P-values were determined by Wilcoxon test for dependent samples following a statistically significant difference as determined by rm-ANOVA, and represent treatment versus baseline. *P<0.05.

Table 3: Mean values by question in an NEM-supplemented treatment group at baseline and 10, 30 and 60 days post-treatment.

osteoarthritis of the knee and/or hip. Of these subjects, twenty-seven (61%) were female and seventeen (39%) were male. The treated joints consisted of knee (39), hip (14), ankle (3), both either knee and hip (10), or both knee and ankle (2). Of the thirty-nine subjects with knee OA, sixteen (40.0%) had bilateral incidence. Of the fourteen subjects with hip OA, seven (50.0%) had bilateral incidence. Patient demographics are reported in Table 1. All forty-four subjects completed baseline evaluations. Thirty-seven (84%) of the forty-four subjects completed the two month study per the protocol. Compliance with the study treatment regimen was good.

Patient data was initially evaluated between sites to exclude site bias (not shown). As there were no abnormalities in these evaluations, the data were pooled for all subsequent analyses. A clinical comparison of valid subjects was carried out to obtain a mean baseline score for each of the ten questions from the patient questionnaire (Table 2). Statistical analysis of the primary outcome measure revealed that supplementation with NEM® produced a significant treatment response from baseline at 10 days (Q1-6 and Q9) (8.6% to 18.1% improvement) and at 30 and

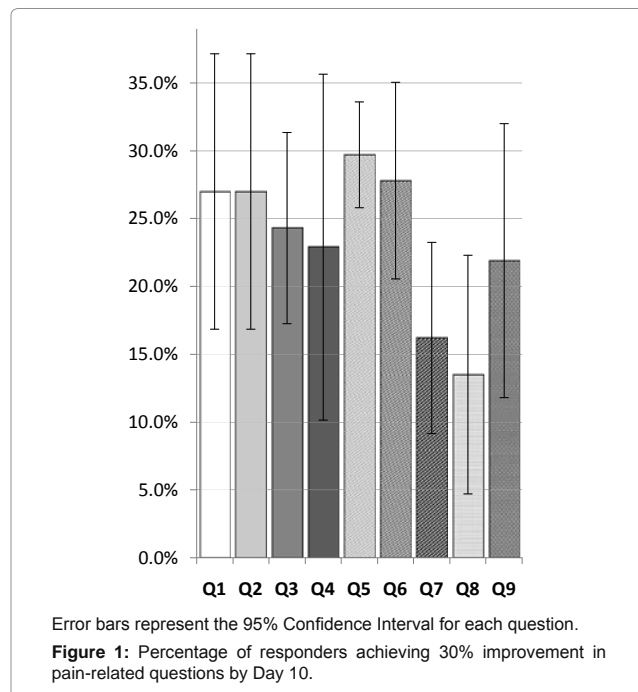
60 days for all nine pain-related questions evaluated (22.4% to 35.6% improvement) (Table 3). Treatment response fell just shy of statistical significance at 10 days for Questions 7 and 8 (p=0.056 and p=0.064, respectively). Supplementation with NEM® produced a significant treatment response from baseline at 30 and 60 days for stiffness (Q10) (27.4% to 29.3% improvement). Greater than 59% of patients rated the efficacy of NEM® as good or very good (Table 4) following 60 days of supplementation. Physicians also rated the treatment effective in subjects, with greater than 75% having moderate or significant improvement from baseline after 60 days (Table 5). For the 30 days prior to study commencement, patients consumed on average 7.0 ± 6.0 doses of acetaminophen. Analgesic use had dropped considerably to 2.43 ± 2.69 doses (per 30 days) at 30 days of supplementation with NEM®. Analgesic use rebounded slightly to 3.59 ± 3.86 doses (per 30 days) by the end of the study at day 60. There were two adverse events reported during the study. One was a scratchy throat and was believed to be related to antibiotic use. The other was stomach discomfort which was believed to be related to the study material. There were no serious

Patient's Global Assessment				
	Efficacy		Tolerability	
	Number	Frequency	Number	Frequency
very good	10	27.0%	22	59.5%
good	12	32.4%	10	27.0%
moderate	9	24.3%	2	5.4%
poor	6	16.2%	3	8.1%

Table 4: Patient's Global Assessment of Efficacy and Tolerability following 60 days of NEM® supplementation.

Physician's Global Assessment		
	Treatment response	
	Number	Frequency
symptom-free	0	0.0%
significant improvement	17	45.9%
moderate improvement	11	29.7%
unchanged	9	24.3%
impaired	0	0.0%

Table 5: Physician's Global Assessment of treatment response following 60 days of NEM® supplementation.



adverse events reported during the study. The treatment was reported to be well tolerated by study participants with greater than 86% of patients rating NEM® tolerability as good or very good

Discussion

Joint and connective tissue disorders are quite common in Westernized countries [1,20] and result in significant costs, both financial [21] and quality-of-life [22], for those that suffer from the debilitating diseases. This open-label clinical trial was designed to evaluate the acceptability of this natural arthritis treatment with European orthopedic surgeons and patients and to validate the

extension of the body of clinical evidence for NEM® from the United States to a European population through the evaluation of the efficacy, safety, and tolerability of NEM® brand eggshell membrane as a treatment option for osteoarthritis of the knee and/or hip. Results of the study indeed suggest that NEM® is both effective and safe for treating pain associated with osteoarthritis of the knee and/or hip in a European population.

Patients experienced relatively rapid (10 days) responses for pain-related questions with a mean response of approximately 14%. By the end of the follow-up period (60 days) the mean response for pain-related questions had more than doubled to approximately 33%. A brief responder analysis of the data provides a number of clinically relevant highlights. On average, nearly 1/4th of the subjects experienced a 30% improvement in pain-related questions within 10 days (Figure 1). And almost 20% of the study population experienced a 50% improvement in pain-related questions by the end of the study (60 days) (not shown). These results align well with results from previous clinical studies of NEM® that were conducted in the U.S. [6,7].

The safety profile for NEM® is also of significance as this is the fifth clinical trial to date in which there have been no reports of serious adverse events associated with treatment. No side effects from consuming NEM® have thus far been identified, excluding the obvious egg allergy concern. This is of obvious importance in a condition such as osteoarthritis that requires long-term treatment.

The trial had a limited initial enrollment (44 subjects), however there was a relatively low drop-out rate (16%) and good treatment compliance. As the trial was also open label, there is the obvious issue of the placebo effect. The inclusion of a placebo control would have provided greater clinical meaning, however it would have required a significantly larger study population.

Conclusions

With so many people suffering from osteoarthritis of the knee and hip in Western populations, it is important for patients to have treatment options that are both safe and effective. The reporting of the results from this six center, open label German clinical study demonstrates that NEM® brand eggshell membrane may be a viable treatment option for the management of osteoarthritis of the knee and/or hip in the broader European population. In this clinical study, NEM®, 500 mg taken once daily, significantly reduced pain, both rapidly (10 days) and continuously (60 days). It also showed clinically meaningful results from a brief responder analysis, demonstrating that a significant proportion of treated patients will benefit from NEM® supplementation.

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References

- Quintana JM, Arostegui I, Escobar A, Azkarate J, Geonaga I, et al (2008) Prevalence of Knee and Hip Osteoarthritis and the Appropriateness of Joint Replacement in an Older Population. Arch Intern Med 168(14): 1576-1584.
- Altman RD (1999) Ibuprofen, acetaminophen and placebo in osteoarthritis of the knee: a six-day double-blind study. Arthritis Rheum 42(S403).
- Case JP, Baliunas AJ, Block JA (2003) Lack of Efficacy of Acetaminophen in Treating Symptomatic Knee Osteoarthritis. Arch Intern Med 163: 169-178.
- Geba GP, Weaver AL, Polis AB, Dixon ME, Schnitzer TJ (2002) Efficacy of

- Rofecoxib, Celecoxib, and Acetaminophen in Osteoarthritis of the Knee. *J Am Med Assoc* 287: 64-71.
5. Towheed TE, Maxwell L, Judd MG, Catton M, Hochberg MC, Wells G (2006) Acetaminophen for osteoarthritis. *Cochrane Database of Systematic Reviews* 1: CD004257.
 6. Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW (2009) Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin Rheumatol* 28: 907-914.
 7. Ruff KJ, DeVore DP, Leu MD, Robinson MA (2009) Eggshell membrane: A possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin Interv Aging* 4: 235-240.
 8. Wong M, Hendrix MJC, von der Mark K, Little C, Stern R (1984) Collagen in the egg shell membranes of the hen. *Dev Biol* 104: 28-36.
 9. Baker JR, Balch DA (1962) A study of the organic material of hen's-egg shell. *Biochem J* 82: 352-361.
 10. Long FD, Adams RG, DeVore DP (2005) Inventors. Preparation of hyaluronic acid from eggshell membrane. U.S. Patent No. 6,946,551.
 11. Ha YW, Son MJ, Yun KS, Kim YS (2007) Relationship between eggshell strength and keratan sulfate of eggshell membranes. *Comp Biochem Physiol A Mol Integr Physiol* 147: 1109-1115.
 12. Richy F, Bruyere O, Ethgen O, Cucherat M, Henrotin Y, Reginster JY (2003) Structural and Symptomatic Efficacy of Glucosamine and Chondroitin in Knee Osteoarthritis: A Comprehensive Meta-analysis. *Arch Intern Med* 163: 1514-1522.
 13. Moreland LW (2003) Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: mechanisms of action. *Arthritis Res Ther* 5: 54-67.
 14. Benson KF, Ruff KJ, Jensen GS (2012) Effects of Natural Eggshell Membrane (NEM) on Cytokine Production in Cultures of Peripheral Blood Mononuclear Cells: Increased Suppression of Tumor Necrosis Factor- α Levels After In Vitro Digestion. *J Med Food* 15: 360-368.
 15. Ruff KJ, DeVore DP (2014) Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product. *Mod Res Inflamm* 3: 19-25.
 16. Baron G, Tubach F, Ravaud P, Logeart I, Dougados M (2007) Validation of a Short Form of the Western Ontario and McMasters Universities Osteoarthritis Index Function Subscale in Hip and Knee Osteoarthritis. *Arthritis Rheum* 57(4): 633-638.
 17. Bilbao A, Quintana JM, Escobar A, Las Hayas C, Orive M (2011) Validation of a proposed WOMAC short form for patients with hip osteoarthritis. *Health Qual Life Outcome* 9: 75.
 18. National Research Council (2003) Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. The National Academies Press Washington, DC.
 19. IBM Corporation. SPSS Statistics.
 20. Helmick CG, Felson DT, Lawrence RC, Gabriel S, Hirsch R, et al (2008) Estimates of the Prevalence of Arthritis and Other Rheumatic Conditions in the United States. Part I. *Arthritis Rheum* 58: 15-25.
 21. U.S. Centers for Disease Control (2007) National and state medical expenditures and lost earnings attributable to arthritis and other rheumatic conditions -United States, 2003. *Morbidity and Mortality Weekly Report* 56: 4-7.
 22. Cook C, Pietrobon R, Hegedus E (2007) Osteoarthritis and the impact on quality of life health indicators. *Rheumatol Int* 27: 315-321.

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Reduction of pro-inflammatory cytokines in rats following 7-day oral supplementation with a proprietary eggshell membrane-derived product

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ABSTRACT

NEM[®] brand eggshell membrane is a novel dietary supplement that has been clinically shown to alleviate arthritis joint pain and stiffness; however the mechanism of action is not well understood. Preliminary evidence from an *in vitro* study of NEM[®] indicated that the mechanism of action may be based on the reduction of pro-inflammatory cytokines. *In vivo* studies were therefore initiated to evaluate the effects of NEM[®] on pro-inflammatory and anti-inflammatory cytokines following oral administration in rats. NEM[®] was administered daily at doses of 6.13 mg/kg bw/day (Study 1), 10.0 mg/kg bw/day (Study 2), or at doses of 0 (control), 26.0 or 52.0 mg/kg bw/day (Study 3) by oral gavage for 7 consecutive days. Inflammation was induced in the Study 3 rats by intraperitoneal injection of lipopolysaccharide. Changes in plasma cytokine levels from baseline following 7 days of oral supplementation with NEM[®] at 6.13 mg/kg bw/day (Study 1) were statistically significant at Day 8 for IL-2, TIMP-1 and VEGF, at Day 21 for IL-10, and at Day 35 for MCP-1, MCP-3 and TIMP-1, and at 10.0 mg/kg bw/day (Study 2) were statistically significant at Day 8 for VEGF, at Day 21 for MIP-1 β , MIP-2 and VEGF, and at Day 35 for MCP-3, MIP-1 β , MIP-2 and VEGF. Changes in serum cytokine levels versus control at 26.0 mg/kg bw/day (Study 3) were statistically significant at all time-points for IL-1 β and at 1.5 hours for IL-10, and at 52.0 mg/kg bw/day (Study 3) were statistically significant at 1.5 hours for IFN- γ , IL-1 β and IL-10, and at 3 hours for IL-1 β ,

and at 24 hours for IL-10. Taken together, these studies demonstrate that oral supplementation with NEM[®] can influence both early-phase pro-inflammatory cytokines like IL-1 β and TNF- α (Study 3), as well as later-phase cytokines like MCP-1, MIP-1 α & β , RANTES and VEGF (Study 1 & 2). These studies provide a possible basis for the mechanism of action of NEM[®] *in vivo*.

KEYWORDS

Pro-Inflammatory Cytokines; Eggshell Membrane

1. INTRODUCTION

Many human diseases are characterized by chronic inflammation which ultimately leads to tissue destruction. Inflammatory arthritides like rheumatoid arthritis (RA) and osteoarthritis (OA) are classic examples of such diseases and the roles that inflammatory chemokines and cytokines play in the pathogenesis of these diseases are fairly well accepted [1-6]. Corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ibuprofen, diclofenac, celecoxib), and inflammatory cytokine-specific biologics (e.g. etanercept, infliximab, adalimumab) are commonly prescribed to address the underlying inflammation of these debilitating conditions. While some of these treatments have demonstrated good efficacy in randomized controlled clinical trials (RCTs), they are also known to have significant and sometimes severe side effects [7-10]. NEM[®] brand eggshell membrane has previously demonstrated good efficacy in relieving pain and stiffness associated with OA of the knee in an RCT [11] and has shown similar efficacy in limited trials for

other affected joints [12,13] with no reports of any significant side effects during these trials.

Eggshell membrane is primarily composed of fibrous proteins such as Collagen Type I [14]. However, eggshell membranes have also been shown to contain other bioactive components, namely glycosaminoglycans (e.g. dermatan sulfate [15], chondroitin sulfate [15], hyaluronic acid [16], etc.). ESM Technologies, LLC (Carthage, MO USA) has developed methods to efficiently and effectively separate eggshell membrane from eggshells on a commercial metric-ton scale. The isolated membrane is then partially hydrolyzed using a proprietary process and dry-blended to produce NEM[®] brand eggshell membrane. Compositional analysis of NEM[®] conducted by ESM has identified a high content of protein and moderate quantities of glucosamine (up to 1% by dry weight), chondroitin sulfate (up to 1%), hyaluronic acid (up to 2%), and collagen (Type I, up to 5%).

Although NEM[®] has been clinically shown to alleviate joint pain and stiffness from arthritis, the mechanism of action of this eggshell membrane preparation is not well understood. Preliminary evidence from an *in vitro* study of NEM[®] indicated that the basis for the mechanism of action may be via the reduction of pro-inflammatory cytokines [17]. *In vivo* studies were therefore initiated to evaluate the effects of NEM[®] on pro-inflammatory and anti-inflammatory cytokines following oral administration in rats. The results of these preliminary studies are reported herein.

2. MATERIALS AND METHODS

2.1. Animals, Care and Diet

Studies were conducted utilizing the facilities and staff of Charles River Laboratories (Spencerville, OH) (Study 1 & 2) and Ricerca Biosciences (Taipei, Taiwan) (Study 3). Animals were housed and cared for in general accordance with the "Guide for the Care and Use of Laboratory Animals" (National Academies Press, Washington, D.C. USA, 1996). Male Sprague Dawley rats were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN USA (Study 1 & 2) and BioLASCO Taiwan Co., Ltd. (a Charles River licensee), Taipei, Taiwan (Study 3) at approximately 7 - 10 weeks of age. Upon receipt, tags with unique identification numbers were used to individually identify the animals. Cage cards displaying the study number, animal number, and sex were affixed to each cage. The rats were acclimatized for 5 days prior to study commencement and were observed daily for overt physical or behavioral abnormalities, general health/morbidity, and mortality. Healthy rats weighing 300 ± 20 g were randomized into groups of three and were housed in cages under standard experimental conditions (22°C \pm 3°C; 30% - 70% humidity; 12-hour light/dark cycle;

minimum 10 room air changes per hour) and had access to standard rat chow [PMI Certified Rodent Chow #5002 (PMI Nutrition International, St. Louis, MO USA) (Study 1 & 2) or Laboratory Rodent Diet MF-18 (Oriental Yeast Co., Ltd., Tokyo, Japan)(Study 3)] and water *ad libitum*.

2.2. Test Article Preparation and Dosing

The test article was prepared by suspending NEM[®] powder (ESM Technologies, LLC, Carthage, MO USA) in 0.5% methylcellulose (Sigma-Aldrich, St. Louis, MO USA) in distilled water at a concentration of 0.613 mg/mL (Study 1), 1.0 mg/mL (Study 2), or 2.6 mg/mL and 5.2 mg/mL (Study 3), corresponding to a dose volume of 10 mL/kg. The test article was stored at approximately 4°C with constant stirring between daily uses. The NEM[®] suspension was administered daily to groups of 3 rats (Study 1 & 2) or groups of 8 rats (Study 3) at doses of 6.13 mg/kg bw/day (Study 1), 10.0 mg/kg bw/day (Study 2), or at doses of 0 (control, vehicle only), 26.0 mg/kg bw/day, or 52.0 mg/kg bw/day (Study 3) by oral gavage for 7 consecutive days. The rats were observed twice daily following administration of the test article for mortality and clinical abnormalities during the study period.

2.3. Induction of Inflammation (Study 3)

Inflammation was induced in the Study 3 rats by intraperitoneal (i.p.) injection (2.5 mg/kg) of a solution of lipopolysaccharide (LPS) (*E. coli* serotype 055:B5, Sigma-Aldrich, St. Louis, MO USA) in pyrogen free saline (Taiwan Biotech Co., Ltd., Taoyuan, Taiwan). Control rats received an i.p. injection of saline only.

2.4. Blood Collection and Cytokine Measurement

Blood samples (~0.5 mL) were collected via jugular vein (Study 1 & 2) or tail vein (Study 3) pre-dose (Day 0) (all studies) and on Days 8, 21 and 35 (Study 1 & 2) or 1.5, 3 and 24 hours post LPS injection (Study 3). Blood samples were processed at the time of collection into plasma samples (Study 1 & 2) or serum samples (Study 3) and were stored at -70°C until cytokine determination could be performed.

Cytokine determination for Study 1 & 2 was accomplished utilizing the facilities and services of Rules Based Medicine, Inc., Austin, TX USA using their Rodent MAP[®] multi-analyte profile platform following the manufacturer's instructions. In this instance, the levels of 42 different biomarkers, chemokines and cytokines were evaluated, however only 16 of these which are related to inflammation [Granulocyte Chemotactic Protein 2 (GCP-2), Interferon gamma (IFN- γ), Interleukin 1 beta (IL-1 β),

IL-2, IL-4, IL-6, IL-10, Monocyte Chemotactic Protein 1 (MCP-1), MCP-3, Macrophage Inflammatory Protein 1 alpha (MIP-1 α), MIP-1 β , MIP-2, Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES), Tissue Inhibitor of Metalloproteinase Type 1 (TIMP-1), Tumor Necrosis Factor alpha (TNF- α), and Vascular Endothelial Growth Factor (VEGF) are reported herein. Cytokine determination for Study 3 was performed by Ricerca Biosciences, LLC, Bothell, WA USA using the Luminex xMAP[®] bead-based multiplex platform (Austin, TX USA) following the manufacturer's instructions. In this instance, the levels of 5 chemokines/cytokines related to inflammation (IFN- γ , IL-1 β , IL-6, IL-10, TNF- α) were evaluated and are reported herein.

2.5. Statistical Analysis

Comparisons of baseline data between groups (Study 3) were made with a Kruskal-Wallis test for multiple independent samples to validate randomization. Statistical significance was accepted at an α value of < 0.05 . Post-baseline statistical analyses were done as repeated measures univariate Analysis of Variance (rm-ANOVA) versus baseline (Study 1 & 2) or versus control (Study 3). Items found to have statistical significance with rm-ANOVA were then compared using a post hoc test for repeated measures. Statistical significance was accepted at an α value of < 0.05 for both determinations. In cases where post-baseline cytokine values were below the Limit of Quantitation (LOQ), a value of $\frac{1}{2}$ LOQ (well above the Limit of Detection for the assays) was incorporated for statistical calculations as opposed to incorporating zero values (Study 1 & 2). This substitution approach was developed in consultation with the assay manufacturer and cases where this approach was used are denoted in the data tables. Additionally, data points were excluded in cases where there was $>35\%$ variance between replicates (Study 3). This occurred at a rate of $<4\%$ in the overall dataset and appeared to be randomly distributed throughout. SYSTAT software (version 13) was used for all statistical analyses [18].

3. RESULTS

Changes in plasma cytokine levels from baseline following 7 days of oral supplementation with NEM[®] at 6.13 mg/kg bw/day (Study 1, Table 1) were statistically significant at Day 8 for IL-2 (153% increase, $p = 0.033$), TIMP-1 (11.2% reduction, $p = 0.002$) and VEGF (27.8% reduction, $p = 0.022$), at Day 21 for IL-10 (65.1% reduction, $p = 0.033$), and at Day 35 for MCP-1 (30.0% reduction, $p = 0.034$), MCP-3 (26.6% reduction, $p = 0.007$) and TIMP-1 (14.6% reduction, $p = 0.032$). There were non-detectable levels of MIP-1 β at Day 21 and MIP-2 and TNF- α at Day 35.

Table 1. Change in plasma cytokine levels from baseline in healthy rats following 7 days of oral supplementation with NEM[®] at 6.13 mg/kg bw/day.

	Baseline (Day 0)	NEM (Day 8)	NEM (Day 21)	NEM (Day 35)
	n = 3	n = 3	n = 3	n = 3
GCP-2 ^a	0.07 \pm 0.02	0.22 \pm 0.10	0.24 \pm 0.07 [†]	0.06 \pm 0.02
IFN- γ ^b	< LOQ	< LOQ	< LOQ	< LOQ
IL-1 β ^a	1.29 \pm 0.99	0.72 \pm 0.34	0.62 \pm 0.22	0.30 \pm 0.12 ^c
IL-2 ^b	11.2 \pm 6.2	28.3 \pm 9.0 [*]	33.9 \pm 23.6	26.0 \pm 13.0
IL-4 ^b	< LOQ	< LOQ	< LOQ	< LOQ
IL-6 ^b	< LOQ	< LOQ	< LOQ	< LOQ
IL-10 ^b	616 \pm 98	503 \pm 85	215 \pm 105 [*]	477 \pm 62
MCP-1 ^b	457 \pm 65	449 \pm 91	553 \pm 144	320 \pm 101 [*]
MCP-3 ^b	222 \pm 51	207 \pm 39	256 \pm 70	163 \pm 42 [*]
MIP-1 α ^a	0.26 \pm 0.05	0.17 \pm 0.08	0.15 \pm 0.03	0.24 \pm 0.06
MIP-1 β ^b	28.7 \pm 11.5	44.2 \pm 9.1	39.0 \pm 0.0 ^c	27.4 \pm 17.0
MIP-2 ^b	3.0 \pm 0.7	3.3 \pm 0.7	3.6 \pm 1.2	3.6 \pm 0.0 ^c
RANTES ^b	86.2 \pm 36.6	152 \pm 92	319 \pm 70 [†]	62.0 \pm 22.5
TIMP-1 ^a	8.9 \pm 1.4	7.9 \pm 1.3 [*]	8.3 \pm 0.6	7.6 \pm 1.1 [*]
TNF- α ^a	0.05 \pm 0.03	0.05 \pm 0.04 ^c	0.05 \pm 0.04 ^c	0.07 \pm 0.0 ^c
VEGF ^b	227 \pm 38	164 \pm 40 [*]	196 \pm 55	208 \pm 30

Values represent means \pm standard deviation, a = ng/mL, b = pg/mL, <LOQ = below limit of quantitation, P -values determined by repeated measures ANOVA versus baseline, ^{*} $P < 0.05$, [†] $P < 0.10$, c = contained cases where values measured were below the Limit of Quantitation (LOQ) wherein values of $\frac{1}{2}$ LOQ were incorporated for statistical calculations.

Changes in plasma cytokine levels versus baseline following 7 days of oral supplementation with NEM[®] at 10.0 mg/kg bw/day (Study 2, Table 2) were statistically significant at Day 8 for VEGF (50.1% reduction, $p = 0.038$), at Day 21 for MIP-1 β (84.8% reduction, $p = 0.022$), MIP-2 (77.1% reduction, $p = 0.005$) and VEGF (61.5% reduction, $p = 0.014$), and at Day 35 for MCP-3 (67.2% reduction, $p = 0.047$), MIP-1 β (88.4% reduction, $p = 0.002$), MIP-2 (76.5% reduction, $p = 0.006$) and VEGF (66.4% reduction, $p = 0.002$). There were trends toward significance at Day 8 for MIP-2 (64.0% reduction, $p = 0.063$), at Day 21 for MCP-3 (61.9% reduction, $p = 0.081$) and TNF- α (70.0% reduction, $p = 0.097$), and at Day 35 for GCP-2 (53.8% reduction, $p = 0.075$), IL-2 (69.0% reduction, $p = 0.098$) and MCP-1 (67.3% reduction, $p = 0.079$). There were non-detectable levels of IL-2, MIP-2 and TIMP-1 at Day 35.

There were no differences in serum cytokine levels between groups (control, 26.0 mg/kg bw/day or 52.0 mg/kg bw/day) at baseline for any of the five cytokines evaluated (IFN- γ , IL-1 β , IL-6, IL-10, TNF- α) (Study 3, Table 3). Changes in serum cytokine levels versus control following 7 days of oral supplementation with NEM[®] at 26.0 mg/kg bw/day (Study 3, Table 4) with

Table 2. Change in plasma cytokine levels from baseline in healthy rats following 7 days of oral supplementation with NEM[®] at 10.0 mg/kg bw/day.

	Baseline (Day 0)	NEM (Day 8)	NEM (Day 21)	NEM (Day 35)
	n = 3	n = 3	n = 3	n = 3
GCP-2 ^a	0.13 ± 0.05	0.06 ± 0.04	0.02 ± 0.03	0.06 ± 0.02 [†]
IFN- γ ^b	< LOQ	< LOQ	< LOQ	< LOQ
IL-1 β ^a	< LOQ	< LOQ	< LOQ	< LOQ
IL-2 ^b	108 ± 44	31.3 ± 21.3	25.3 ± 14.1 ^c	33.5 ± 0.0 ^{c†}
IL-4 ^b	< LOQ	< LOQ	< LOQ	< LOQ
IL-6 ^b	< LOQ	< LOQ	< LOQ	< LOQ
IL-10 ^b	460 ± 265	305 ± 15	314 ± 25	294 ± 83
MCP-1 ^b	721 ± 233	439 ± 113	250 ± 122	236 ± 28 [†]
MCP-3 ^b	354 ± 93	226 ± 72	135 ± 44 [†]	116 ± 6 [*]
MIP-1 α ^a	< LOQ	< LOQ	< LOQ	< LOQ
MIP-1 β ^b	250 ± 31	81 ± 103	38 ± 46 [*]	29 ± 16 [*]
MIP-2 ^b	15.3 ± 1.5	5.5 ± 3.4 ^{c†}	3.5 ± 0.2 ^{c*}	3.6 ± 0.0 ^{c*}
RANTES ^b	195 ± 118	93 ± 100	23 ± 20	20 ± 4
TIMP-1 ^a	0.28 ± 0.16	0.09 ± 0.06 ^c	0.08 ± 0.03 ^c	0.09 ± 0.00 ^c
TNF- α ^a	0.20 ± 0.09	0.06 ± 0.06	0.06 ± 0.03 ^{c†}	0.04 ± 0.03 ^c
VEGF ^b	429 ± 42	214 ± 75 [*]	165 ± 25 [*]	144 ± 33 [*]

Values represent means ± standard deviation, a = ng/mL, b = pg/mL, < LOQ = below limit of quantitation, *P*-values determined by repeated measures ANOVA versus baseline, **P* < 0.05, [†]*P* < 0.10, c = contained cases where values measured were below the Limit of Quantitation (LOQ) wherein values of 1/2LOQ were incorporated for statistical calculations.

Table 3. Mean serum cytokine concentrations (pg/mL) in NEM-supplemented and control groups at baseline.

	Control (0 mg/kg)	NEM (26 mg/kg)	NEM (52 mg/kg)
	n = 8	n = 8	n = 8
IFN- γ	2.49 ± 0.16	2.40 ± 0.00	2.49 ± 0.27
IL-1 β	15.8 ± 10.0	13.0 ± 5.5	18.6 ± 13.2
TNF- α	11.1 ± 2.0	10.2 ± 0.7	11.2 ± 2.9
IL-6	9.80 ± 0.00	9.96 ± 1.14	10.2 ± 1.1
IL-10	10.2 ± 1.0	9.80 ± 0.00	10.0 ± 0.6

Values represent means ± standard deviation, *P*-values determined by Kruskal-Wallis test for multiple independent samples, **P* < 0.05, [†]*P* < 0.10.

subsequent inflammatory challenge (LPS, i.p.) were statistically significant at 1.5 hours (43.7% reduction, *p* = 0.013), 3 hours (28.8% reduction, *p* = 0.034) and 24 hours (20.8% reduction, *p* = 0.006) for IL-1 β and at 1.5 hours (27.6% reduction, *p* = 0.028) for IL-10. There was a trend toward significance at 24 hours for IL-10 (74.6% increase, *p* = 0.097). No other changes in serum cytokine levels were statistically significant at this dose level.

Changes in serum cytokine levels versus control following 7 days of oral supplementation with NEM[®] at

52.0 mg/kg bw/day (Study 3, Table 5) with subsequent inflammatory challenge (LPS, i.p.) were statistically significant at 1.5 hours for IFN- γ (33.5% reduction, *p* = 0.047), IL-1 β (39.4% reduction, *p* = 0.003) and IL-10 (29.8% reduction, *p* = 0.015), and at 3 hours for IL-1 β (23.9% reduction, *p* = 0.044), and at 24 hours for IL-10 (57.5% increase, *p* = 0.021). There was a trend toward significance at 24 hours for IL-1 β (9.3% reduction, *p* = 0.093). No other changes in serum cytokine levels were statistically significant at this dose level.

4. DISCUSSION

Although OA has not traditionally been considered an inflammatory arthropathy, the scientific understanding of the pathophysiological progression of the disease has been gradually trending towards that of a disease involving the “whole joint” with significant localized inflammation [19]. Evidence of an inflammatory process in OA is reflected in many of the clinical symptoms of the progressive disease, including swelling of affected joints,

Table 4. Change in mean serum cytokine concentrations (pg/mL) in 7-day NEM-supplemented (26 mg/kg bw/day) and control groups from baseline at 1.5, 3, and 24 hours post LPS treatment.

	Hours post-treatment	Control (0 mg/kg) n = 8	NEM (26 mg/kg) n = 8	% Difference (NEM-vs-ctrl)
IFN- γ	Baseline	2.49 ± 0.16	2.40 ± 0.00	-3.5
	1.5	6.35 ± 2.48	4.88 ± 2.60	-23.2
	3	135 ± 43	144 ± 85	6.2
	24	3.55 ± 2.18	2.49 ± 0.14	-29.8
IL-1 β	Baseline	15.8 ± 10.0	13.0 ± 5.5	-17.9
	1.5	62.4 ± 17.1	35.1 ± 16.7 [*]	-43.7 [*]
	3	87.9 ± 23.5	62.6 ± 15.9 [*]	-28.8 [*]
	24	12.6 ± 2.1	9.96 ± 0.42 [*]	-20.8 [*]
TNF- α	Baseline	11.1 ± 2.0	10.2 ± 0.7	-7.6
	1.5	1157 ± 828	934 ± 332	-19.3
	3	84.5 ± 46.7	75.5 ± 28.7	-10.7
	24	9.80 ± 0.00	9.80 ± 0.00	0.0
IL-6	Baseline	9.80 ± 0.00	9.96 ± 1.14	1.7
	1.5	321 ± 175	385 ± 182	20.0
	3	386 ± 172	329 ± 133	-14.8
	24	10.0 ± 0.6	10.7 ± 2.3	6.6
IL-10	Baseline	10.2 ± 1.0	9.80 ± 0.00	-3.8
	1.5	42.7 ± 12.3	30.9 ± 8.3 [*]	-27.6 [*]
	3	18.7 ± 5.6	19.3 ± 7.4	3.1
	24	14.2 ± 3.2	24.9 ± 11.5 [†]	74.6 [†]

Values represent means ± standard deviation. *P*-values determined by repeated measures ANOVA, **p* < 0.05, [†]*p* < 0.10. ctrl = control.

Table 5. Mean serum cytokine concentrations (pg/mL) in 7-day NEM-supplemented (52 mg/kg bw/day) and control groups at baseline and 1.5, 3, and 24 hours post LPS treatment.

	Hours post-treatment	Control (0 mg/kg) n = 8	NEM (52 mg/kg) n = 8	% Difference (NEM-vs-ctrl)
IFN- γ	Baseline	2.49 \pm 0.16	2.49 \pm 0.27	0.3
	1.5	6.35 \pm 2.48	4.22 \pm 0.76*	-33.5*
	3	135 \pm 43	130 \pm 34	-4.3
	24	3.55 \pm 2.18	2.82 \pm 0.75	-20.7
IL-1 β	Baseline	15.8 \pm 10.0	18.6 \pm 13.2	17.3
	1.5	62.4 \pm 17.1	37.8 \pm 9.7*	-39.4*
	3	87.9 \pm 23.5	66.9 \pm 15.8*	-23.9*
	24	12.6 \pm 2.1	11.4 \pm 1.2	-9.3 [†]
TNF- α	Baseline	11.1 \pm 2.0	11.2 \pm 2.9	1.5
	1.5	1157 \pm 828	786 \pm 161	-32.1
	3	84.5 \pm 46.7	70.0 \pm 15.0	-17.2
	24	9.80 \pm 0.00	9.80 \pm 0.00	0.0
IL-6	Baseline	9.80 \pm 0.00	10.2 \pm 1.1	4.0
	1.5	321 \pm 175	256 \pm 83	-20.1
	3	386 \pm 172	306 \pm 64	-20.6
	24	10.0 \pm 0.6	10.3 \pm 1.4	3.0
IL-10	Baseline	10.2 \pm 1.0	10.0 \pm 0.6	-1.8
	1.5	42.7 \pm 12.3	30.0 \pm 8.1*	-29.8*
	3	18.7 \pm 5.6	18.1 \pm 3.4	-3.6
	24	14.2 \pm 3.2	22.4 \pm 6.1*	57.5*

Values represent means \pm standard deviation. *P*-values determined by repeated measures ANOVA, **p* < 0.05, [†]*p* < 0.10. ctrl = control.

synovial effusion, and joint stiffness [20]. This clinical evidence is supported by immunochemical and histological data from numerous studies showing infiltration of the joint synovium by immune cells, primarily macrophages and mononuclear lymphocytes such as T-cells [20-22] accompanied by subsequent inflammatory cytokine expression [23] and synovial fibroblast activation [24].

The two primary mediators of arthritis inflammation are IL-1 β and TNF- α . These cytokines have been identified as targets for OA treatment [25,26] and there are multiple FDA-approved biologic drugs (etanercept, infliximab, adalimumab, etc.) for this indication (mostly RA). These cytokines, in an autocrine/paracrine manner, auto-amplify their own expression and induce chondrocytes to produce matrix metalloproteinases (MMPs), chemokines (IL-8, MCP-1, MIP-1 α , MIP-1 β , RANTES, etc.), nitric oxide, and prostaglandins [19,26]. This leads to localized tissue destruction, immune cell infiltration, inhibition of cartilage matrix synthesis, and increased pain sensitivity, among others.

The eggshell membrane derived product NEM[®] has previously been shown *in vitro* to reduce a number of pro-inflammatory cytokines in human immune cells following inflammatory challenge (with phyto-mitogens), with this effect being most pronounced for IFN- γ and TNF- α [17]. In this paper, we reported *in vivo* support for the reduction of circulating pro-inflammatory cytokines following oral supplementation with NEM[®] in both healthy rats (Study 1 & 2) and inflammatory-challenged rats (Study 3).

While not statistically significant, NEM[®] appeared to demonstrate trends toward reduction in healthy rats for both IL-1 β (6.13 mg/kg bw/day)(Study 1) and TNF- α (10.0 mg/kg bw/day)(Study 2). Interestingly, there were statistically significant effects at both dose levels for nearly all of the chemokines (MCP-1, MIP-1 α , MIP-1 β , RANTES, VEGF) currently understood to be key players in OA/RA inflammation and pathogenesis. MCP-1 and RANTES have been shown to induce expression of MMP-3 in both normal and OA chondrocytes [27] and RANTES has been reported to stimulate MMP-1 release in chondrocytes as effectively as did IL-1 β [28]. These enzymes are known to degrade chondrocyte extracellular matrix (ECM) which leads to cartilage destruction. MCP-1, RANTES, MIP-1 α and MIP-1 β have all been shown to inhibit proteoglycan synthesis in chondrocytes [27,29], a key component of cartilage needed for repair. VEGF expression is absent in adult healthy cartilage but is significantly expressed in OA chondrocytes and may play a role in osteophyte formation [30].

Also interesting is the overall lack of effect from oral supplementation with NEM[®] on anti-inflammatory cytokines and chemokines (IL-4, IL-6, IL-10, and TIMP-1) in healthy rats. While IL-4 and IL-6 were below LOQ at baseline, there was only a mild downward trend in IL-10 and TIMP-1 levels—consistent with restoring immune homeostasis following the reductions seen in pro-inflammatory cytokines and chemokines. IL-10 is known to inhibit the production of IL-1 β and TNF- α and is overexpressed in OA chondrocytes compared to normal, which is likely the body's attempt to counteract the detrimental effects from these pro-inflammatory cytokines [31]. MMPs are strictly controlled by TIMPs under normal conditions and an imbalance toward MMPs is believed to be the basis for cartilage destruction via ECM degradation in arthritis [32].

NEM[®] has been shown in clinical trials to have an effective dose of 500 mg per day. We initially chose to evaluate doses of 6.13 mg/kg bw/day (Study 1) and 10.0 mg/kg bw/day (Study 2) which, following allometric conversion [33], equate to a human equivalent dose (HED) of 59 mg/day and 97 mg/day, respectively, for a 60 kg person. The number of animals (n = 3) was also small in the preliminary evaluations of inflammatory

cytokines. These facts, combined with low basal cytokine levels in healthy rats, made it challenging to obtain statistically significant changes following oral supplementation with NEM[®]. In a number of instances (IL-1 β in Study 1 and IL-10 & RANTES in Study 2), there appeared to be substantial percent reductions in mean cytokine levels that nevertheless failed to reach statistical significance. We therefore set out to employ a rat model in which inflammation was induced (Study 3) to increase the likelihood of observing clearer effects from NEM[®] supplementation. We also increased the number of animals (n = 8), narrowed the number of cytokines evaluated to five, and increased the doses evaluated to 26.0 mg/kg bw/day and 52.0 mg/kg bw/day (HED: 252 mg/day & 503 mg/day, respectively).

There was a substantial (39% - 44%) and lasting (through 24 hours) reduction in IL-1 β in this inflammatory-challenge model (Study 3) at both doses evaluated. And, although not statistically significant, there also appeared to be a substantial (19% - 32%) downward trend in TNF- α levels for both doses, as well. These effects on the key mediators of arthritis inflammation provide further supportive evidence to the observed clinical efficacy of NEM[®]. The ability to influence IL-1 β and TNF- α *in vivo* likely also explains at least some of the effects observed in the downstream chemokines (MCP-1, MIP-1 α , MIP-1 β , RANTES, VEGF) in the initial studies. Interestingly, there was a sinusoidal response for the anti-inflammatory cytokine IL-10 over the time-course of this study, in which there was an initial substantial reduction (28% - 30%) at 1.5 hours leading to a substantial increase (58% - 75%) by 24 hours when compared to controls. This is particularly interesting in the context that nearly all of the cytokines evaluated had returned to near baseline levels by the 24-hour study endpoint in the control animals. The reason for this divergence isn't completely clear, but it may be a result of the delayed time-course of anti-inflammatory cytokines compared to the rapid time-course of pro-inflammatory cytokines, especially in this particular animal model.

Taken together, these studies demonstrate that oral supplementation with NEM[®] can influence both early-phase pro-inflammatory cytokines like IL-1 β and TNF- α (Study 3), as well as later-phase pro-inflammatory cytokines like MCP-1, MIP-1 α & β , RANTES and VEGF (Study 1 & 2). There was also a mild effect on the anti-inflammatory cytokine IL-10 in all three studies. A natural treatment, such as NEM[®], which is suitable for chronic inflammatory diseases like arthritis that could potentially avoid the unfortunate side effects of currently available pharmaceutical treatments is of obvious benefit. These studies provide a possible basis for the mechanism of action of NEM[®] *in vivo* and serve as an important step in explaining its observed clinical efficacy seen in mul-

tiple human studies.

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REFERENCES

- [1] Harrington, J.J., Ludikhuizen, J. and Tak, P.P. (2004) Chemokines in joint disease: The key to inflammation? *Annals of the Rheumatic Diseases*, **63**, 1186-1194. <http://dx.doi.org/10.1136/ard.2004.020529>
- [2] Martel-Pelletier, J., Alaaeddine, N. and Pelletier, J.P. (1999) Cytokines and their role in the pathophysiology of osteoarthritis. *Frontiers in Bioscience*, **4**, d694-d703. <http://dx.doi.org/10.2741/Martel>
- [3] Choy, E.H.S. and Panayi, G.S. (2001) Cytokine pathways and joint inflammation in rheumatoid arthritis. *New England Journal of Medicine*, **344**, 907-916. <http://dx.doi.org/10.1056/NEJM200103223441207>
- [4] Feldmann, M. and Maini, R.N. (2008) Role of cytokines in rheumatoid arthritis: An education in pathophysiology and therapeutics. *Immunological Reviews*, **223**, 7-19. <http://dx.doi.org/10.1111/j.1600-065X.2008.00626.x>
- [5] Brennan, F.M. and McInnes, I.B. (2008) Evidence that cytokines play a role in rheumatoid arthritis. *The Journal of Clinical Investigations*, **118**, 3537-3545. <http://dx.doi.org/10.1172/JCI36389>
- [6] Kokkonen, H., Soderstrom, I., Rocklov, J., Hallmans, G., Lejon, K. and Dahlqvist, S.R. (2010) Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. *Arthritis & Rheumatism*, **62**, 383-391. <http://dx.doi.org/10.1002/art.27186>
- [7] Dixon, W.G., Suissa, S. and Hudson, M. (2011) The association between systemic glucocorticoid therapy and the risk of infection in patients with rheumatoid arthritis: Systematic review and meta-analyses. *Arthritis Research & Therapy*, **13**, R139. <http://dx.doi.org/10.1186/ar3453>
- [8] Singh, G., Wu, O., Langhorne, P. and Madhok, R. (2006) Risk of acute myocardial infarction with nonselective non-steroidal anti-inflammatory drugs: A meta-analysis. *Arthritis Research & Therapy*, **8**, 153-162. <http://dx.doi.org/10.1186/ar2047>
- [9] Masso Gonzalez, E.L., Patrignani, P., Tacconelli, S. and Garcia Rodriguez, L.A. (2010) Variability among non-steroidal antiinflammatory drugs in risk of upper gastrointestinal bleeding. *Arthritis & Rheumatism*, **62**, 1592-1601. <http://dx.doi.org/10.1002/art.27412>
- [10] O'Neil, C.K., Hanlon, J.T. and Marcum, Z.A. (2012) Adverse effects of analgesics commonly used by older adults with osteoarthritis—Focus on non-opioid and opioid analgesics. *American Journal of Geriatric Pharmacotherapy*, **10**, 331-342. <http://dx.doi.org/10.1016/j.amjopharm.2012.09.004>
- [11] Ruff, K.J., Winkler, A., Jackson, R.W., DeVore, D.P. and Ritz, B.W. (2009) Eggshell membrane in the treatment of

- pain and stiffness from osteoarthritis of the knee: A randomized, multicenter, double-blind, placebo-controlled clinical study. *Clinical Rheumatology*, **28**, 907-914.
<http://dx.doi.org/10.1007/s10067-009-1173-4>
- [12] Ruff, K.J., DeVore, D.P., Leu, M.D. and Robinson, M.A. (2009) Eggshell membrane: A possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clinical Interventions in Aging*, **4**, 235-240.
<http://dx.doi.org/10.2147/CIA.S5797>
- [13] Danesch, U., Seybold, M., Rittinghausen, R., Treibel, W. and Bitterlich, N. (Unpublished) NEM[®] brand eggshell membrane effective in the treatment of pain associated with knee and hip osteoarthritis: Results from a six-center, open-label german clinical study.
- [14] Wong, M., Hendrix, M.J.C., von der Mark, K., Little and C., Stern, R. (1984) Collagen in the egg shell membranes of the hen. *Developmental Biology*, **104**, 28-36.
[http://dx.doi.org/10.1016/0012-1606\(84\)90033-2](http://dx.doi.org/10.1016/0012-1606(84)90033-2)
- [15] Baker, J.R. and Balch, D.A. (1962) A study of the organic material of hen's-egg shell. *Biochemical Journal*, **82**, 352-361.
- [16] Long, F.D., Adams, R.G., DeVore, D.P., Inventors (2005) Preparation of hyaluronic acid from eggshell membrane. US Patent No. 6,946,551.
- [17] Benson, K.F., Ruff, K.J. and Jensen, G.S. (2012) Effects of natural eggshell membrane (nem) on cytokine production in cultures of peripheral blood mononuclear cells: increased suppression of tumor necrosis factor- α levels after in vitro digestion. *Journal of Medicinal Food*, **15**, 360-368. <http://dx.doi.org/10.1089/jmf.2011.0197>
- [18] Systat Software, Inc. <http://www.systat.com>
- [19] Abramson, S.B. and Attur, M. (2009) Developments in the scientific understanding of osteoarthritis. *Arthritis Research & Therapy*, **11**, 227.
<http://dx.doi.org/10.1186/ar2655>
- [20] Sellam, J. and Berenbaum, F. (2010) The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nature Reviews Rheumatology*, **6**, 625-635.
<http://dx.doi.org/10.1038/nrrheum.2010.159>
- [21] Roach, H.I., Aigner, T., Soder, S., Haag, J. and Welkerling, H. (2007) Pathobiology of osteoarthritis: Pathomechanisms and potential therapeutic targets. *Current Drug Targets*, **8**, 271-282.
<http://dx.doi.org/10.2174/138945007779940160>
- [22] Sakkas, L.I. and Platsoucas, C.D. (2007) The role of T cells in the pathogenesis of osteoarthritis. *Arthritis & Rheumatism*, **56**, 409-424.
<http://dx.doi.org/10.1002/art.22369>
- [23] Bondeson, J., Wainwright, S.D., Lauder, S., Amos, N. and Hughes, C.E. (2006) The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Research & Therapy*, **8**, R187.
<http://dx.doi.org/10.1186/ar2099>
- [24] Wenham, C.Y.J. and Conaghan, P.G. (2010) The role of synovitis in osteoarthritis. *Therapeutic Advances in Musculoskeletal Diseases*, **2**, 349-359.
<http://dx.doi.org/10.1177/1759720X10378373>
- [25] Martel-Pelletier, J., Tardif, G., Laufer, S. and Pelletier, J.P. (2005) Cytokines and growth factors in the treatment of osteoarthritis: What could be the best disease modifying drugs. *Current Medicinal Chemistry—Anti-Inflammatory & Anti-Allergy Agents*, **4**, 235-249.
- [26] Yuan, G.H., Masuko-Hongo, K., Kato, T. and Nishioka, K. (2003) Immunologic intervention in the pathogenesis of osteoarthritis. *Arthritis & Rheumatism*, **48**, 602-611.
<http://dx.doi.org/10.1002/art.10768>
- [27] Yuan, G.H., Masuko-Hongo, K., Sakata, M., et al. (2001) The role of C-C chemokines and their receptors in osteoarthritis. *Arthritis & Rheumatism*, **44**, 1056-1070.
[http://dx.doi.org/10.1002/1529-0131\(200105\)44:5%3C1056::AID-ANR186%3E3.0.CO;2-U](http://dx.doi.org/10.1002/1529-0131(200105)44:5%3C1056::AID-ANR186%3E3.0.CO;2-U)
- [28] Alaaeddine, N., Olee, T., Hashimoto, S., Creighton-Achermann, L. and Lotz, M. (2001) Production of the chemokine RANTES by articular chondrocytes and role in cartilage degradation. *Arthritis & Rheumatism*, **44**, 1633-1643.
[http://dx.doi.org/10.1002/1529-0131\(200107\)44:7%3C1633::AID-ART286%3E3.0.CO;2-Z](http://dx.doi.org/10.1002/1529-0131(200107)44:7%3C1633::AID-ART286%3E3.0.CO;2-Z)
- [29] Vergunst, C.E., van de Sande, M.G.H., Lebre, M.C. and Tak, P.P. (2005) The role of chemokines in rheumatoid arthritis and osteoarthritis. *Scandinavian Journal of Rheumatology*, **34**, 415-425.
<http://dx.doi.org/10.1080/03009740500439159>
- [30] Tanaka, E., Aoyama, J., Miyauchi, M., et al. (2005) Vascular endothelial growth factor plays an important autocrine/paracrine role in the progression of osteoarthritis. *Histochemistry and Cell Biology*, **123**, 275-281.
<http://dx.doi.org/10.1007/s00418-005-0773-6>
- [31] Iannone, F., De Bari, C., Dell'Accio, F., et al. (2001) Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes. *Clinical & Experimental Rheumatology*, **19**, 139-145.
- [32] Ishiguro, N., Ito, T., Ito, H., et al. (1999) Relationship of matrix metalloproteinases and their inhibitors to cartilage proteoglycan and collagen turnover: Analyses of synovial fluid from patients with osteoarthritis. *Arthritis & Rheumatism*, **42**, 129-136.
[http://dx.doi.org/10.1002/1529-0131\(199901\)42:1%3C129::AID-ANR16%3E3.0.CO;2-4](http://dx.doi.org/10.1002/1529-0131(199901)42:1%3C129::AID-ANR16%3E3.0.CO;2-4)
- [33] US Food and Drug Administration (2005) Guidance for industry: estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers.
<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>

Effects of Natural Eggshell Membrane (NEM) on Cytokine Production in Cultures of Peripheral Blood Mononuclear Cells: Increased Suppression of Tumor Necrosis Factor- α Levels After *In Vitro* Digestion

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ABSTRACT Tumor necrosis factor- α (TNF- α) plays an important role in inflammatory processes. This study examined the effects of natural eggshell membrane (NEM[®]) (ESM Technologies, LLC, Carthage, MO, USA) on interleukin (IL)-2, IL-4, IL-6, IL-10, interferon- γ (IFN- γ), and TNF- α cytokine production by 4-day peripheral blood mononuclear cell (PBMC) cultures exposed to serial dilutions of either an aqueous extract of natural eggshell membrane (NEM-AQ) or NEM subjected to *in vitro* digestion (NEM-IVD). The effects on cytokine production were also assessed in the presence of phytohemagglutinin (PHA) and pokeweed mitogen (PWM) where exposure to NEM-AQ resulted in reduced levels of proliferation and statistically significant effects on IL-6, IL-10, IFN- γ , and TNF- α cytokine production. NEM-AQ reduced levels of IL-6, IL-10, IFN- γ , and TNF- α in cultures exposed to PHA. In cultures containing PWM, NEM-AQ reduced production of IL-10 and at the highest dose tested increased IL-6 and decreased TNF- α cytokine levels. NEM-IVD, at the two lowest concentrations of product, significantly reduced TNF- α production by PBMC cultures exposed to PWM compared with the *in vitro* digest control or native NEM. Taken together, these results suggest that NEM-AQ can influence signaling events in response to the T cell-specific mitogen PHA as well as to the mitogen PWM that require cellular cross-talk and that these effects may be partially mediated through a reduction in level of the pro-inflammatory cytokine TNF- α . The suppression of TNF- α production in the presence of NEM-IVD is promising for the use of NEM as a consumable anti-inflammatory product.

KEY WORDS: • cytokines • human • immunity • *in vitro* digestion • lymphocyte • natural eggshell membrane • natural product • peripheral blood mononuclear cells • Th1/Th2 • tumor necrosis factor- α

INTRODUCTION

THE MAIN CLINICAL MANIFESTATIONS of arthritis are inflammation, pain, and bone resorption. Chronic inflammation and bone loss are closely linked pathophysiologic events. New scientific data point to a beneficial effect of blocking specific molecular interactions, which can reduce local arthritic symptoms even in the presence of ongoing chronic inflammation.¹ The current mainstream medical treatments for arthritis involve pain management, anti-inflammatory drugs (nonsteroidal anti-inflammatory drugs, steroids, cyclooxygenase-2 inhibitors), and also exploration of chemokine receptor antagonists to stop cell migration into the inflamed areas.^{2–4} Part of the intensive pharmaceutical research efforts includes research on the interaction between osteoblasts and osteoclasts via the receptor activator of nuclear factor κ B and its ligand. Receptor activator of nuclear factor κ is a hematopoietic surface receptor controlling osteoclastogenesis and calcium metabo-

lism. Interference with these various pathways may also include arresting the maturation of phagocytic mononuclear cells into bone-resorbing cells, neutralizing pro-inflammatory cytokines, and blocking of matrix metalloproteinases. These mainstream treatments go far beyond a direct treatment of cells within the arthritic joints. They aim at reducing inflammation and inhibiting recruitment into the inflamed area of cells that contribute to disease processes, including bone resorption.

In contrast, nutraceutical products widely used for joint health include glucosamine, chondroitin, and hyaluronic acid, thus ignoring a multifaceted action of complex natural products. Even the spotlight on hyaluronic acid seems to limit its focus on replenishing the synovial fluid and on stimulating chondrocytes to produce more hyaluronic acid, thus ignoring the many complex ways that hyaluronic acid can modulate cells and their behavior.

Natural eggshell membrane (NEM[®]) (ESM Technologies, LLC, Carthage, MO, USA) is a novel dietary supplement that has been shown in several human trials to be a clinically effective treatment for pain and stiffness associated with joint and connective tissue disorders, particularly osteoarthritis.^{5,6} Eggshell membrane is primarily composed

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of fibrous proteins such as collagen type I.⁷ However, eggshell membranes have also been shown to contain glycosaminoglycans, such as dermatan sulfate, chondroitin sulfate,⁸ and hyaluronic acid,⁹ and sulfated glycoproteins, including hexosamines such as glucosamine.¹⁰ NEM contains up to about 5% of these various components, the unique combination of which may explain its biological activity. To further this understanding, a cytokine profile was determined from NEM-treated lymphocytes in cell culture.

The purpose of this study is to evaluate anti-inflammatory and immunomodulatory effects of NEM as well as its *in vitro* digest in a select series of human cell-based *in vitro* assays, in preparation for more comprehensive evaluations *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents

Phosphate-buffered saline (PBS) (pH 7.4), RPMI-1640 culture medium, fetal calf serum, L-glutamine (200 mM), penicillin–streptomycin (100× solution), glacial acetic acid, methanol, pepsin, pancreatin, bile salts, Histopaque 1077, and Histopaque 1119 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cytometric bead array (CBA) for human Th1/Th2 cytokine kit II was purchased from BD Biosciences (San Jose, CA, USA). All reagents for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and silver stain detection were obtained from Bio-Rad (Hercules, CA, USA) and included 4–15% Tris-HCl ready gels, Precision Plus dual color protein molecular weight standards, Laemmli buffer, 10×Tris/glycine/SDS buffer, silver stain concentrate, silver stain oxidizer, and silver stain developer. NEM was obtained from ESM Technologies, LLC.

Preparation of NEM for *in vitro* bioassays

The NEM powder was reconstituted in physiological saline and allowed to rehydrate for 1 hour at room temperature. Solids included insoluble calcium carbonate from eggshell and were removed by centrifugation at 900 g for 10 minutes. The liquid was filtered through a sterile cellulose acetate syringe filter (pore size, 0.22 μ m). This filtrate corresponded to a stock solution of 100 g/L product (same concentration as the *in vitro*-digested NEM [NEM-IVD], described below). This aqueous preparation is designated NEM-AQ.

Preparation of an *in vitro* digest of NEM

The *in vitro* digestion of NEM was performed according to methods published in the literature^{11–13} (see also Fig. 4). In brief, 3.75 g of NEM powder was added to 30 mL of PBS and shaken at room temperature for 1 hour. Following the 1-hour incubation, the sample was spun at 900 g for 10 minutes, and the aqueous solution was removed from the solids and sterile-filtered with a cellulose acetate filter (pore size, 0.22 μ m). HCl (1 M) was then added until the solution reached pH 2.0. Porcine pepsin was then added at a con-

centration of 1.3 mg/mL, and the sample was left at 37°C for 60 minutes with shaking (to simulate digestion in the stomach). Next, sodium bicarbonate (NaHCO₃) was used to increase the pH of the solution to 5.8 (irreversibly inactivating pepsin), and pancreatin (0.175 mg/mL) and porcine bile salts (1.1 mg/mL) were added to simulate intestinal digestion. The pH was then adjusted to 6.5, and the mixture was left at 37°C for 1 hour. The final volume of the digest was adjusted with saline to give a final concentration of NEM of 100 g/L. Following this *in vitro* digestion, the sample was centrifuged through a 10-kDa cutoff filtration spin column to remove the enzymes from the digested product. This filtration step was necessary to avoid the presence of digestive enzymes in the downstream treatment of cells with product. This step also avoided the use of enzyme inhibitors that potentially could have direct effects on cell signaling in downstream cell-based assays. The liquid after *in vitro* digestion and size-exclusion filtration is designated NEM-IVD.

As the control, saline alone (negative control) was simultaneously subjected to the *in vitro* digestion protocol as described above. This was an important control to determine whether any bile salts or breakdown products from the enzymes themselves have biological activity. This PBS control is designated as PBS-IVD.

SDS-PAGE

SDS-PAGE was performed to compare crude NEM-AQ, NEM-IVD, and the PBS-IVD control. Samples were denatured by boiling for 3 minutes in 1×Laemmli buffer and separated by gel electrophoresis through a 4–15% polyacrylamide Tris-HCl gel using Protein Plus dual color molecular weight standards for reference. Silver staining was performed in order to visualize proteins, and an image of the stained gel was captured with a Canon (Lake Success, NY, USA) PowerShot SD430 digital camera.

Purification of peripheral blood mononuclear cells

Healthy human volunteers between the ages of 20 and 50 years served as blood donors after written informed consent was obtained, as approved by the Sky Lakes Medical (Klamath Falls, OR, USA) Center Institutional Review Board. Isolation of peripheral blood mononuclear cells (PBMCs) was performed as previously described.¹⁴ PBMCs were used to establish lymphocyte cultures for the measurement of cytokine production.

Cytokine production by 4-day PBMC cultures

Freshly purified PBMCs were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 mg/mL) at a density of 1×10^6 /mL. Cells were cultured in the presence of 10-fold serial dilutions of NEM-AQ, NEM-IVD, or PBS-IVD in a series of triplicate wells containing a total volume of 200 μ L. Three separate sets of

culture conditions were established: no mitogen, phytohemagglutinin (PHA), or pokeweed mitogen (PWM). The culture plate was incubated at 37°C in an atmosphere of 5% CO₂ for 4 days, after which cells were transferred to a V-bottom plate and centrifuged. Cell supernatants were collected for cytokine measurement (described below). Determination of relative cell numbers in each culture well was performed by staining cells with the DNA dye Cy-Quant® (Invitrogen, Carlsbad, CA, USA) and measuring fluorescence measured with a Tecan (Durham, NC, USA) Spectrafluor fluorescence plate reader. Samples were assayed in triplicate, and experiments were repeated three times with cells from three different donors.

Th1/Th2 cytokine profile

The cytokines interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) were quantified in the 4-day PBMC culture supernatants using a flow cytometry cytometric bead array (CBA) kit (CBA human Th1/Th2 cytokine kit II, BD Biosciences) that allowed the determination of the levels of all six cytokines simultaneously. Samples were tested in duplicate following the manufacturer's instructions, data were acquired with a FACSCalibur™ flow cytometer (Becton-Dickinson, San Jose), and the analysis was performed with FlowJo software (TreeStar Inc., Ashland, OR, USA).

Statistical analysis

Statistical significance was tested using Student's *t* test performed with the program Microsoft (Redmond, WA, USA) Excel. All *P* values were two-sided and were considered significant when *P* < .05. Only statistically significant *P* values are reported.

RESULTS

Lymphocyte proliferation assay

The lymphocyte proliferation assay evaluates whether a test product alters lymphocyte responsiveness to known signals such as mitogens. If any change in the proliferative response to known mitogens is seen in cells pretreated with test product, this is a good indication that the product has immunomodulatory effects and justifies further in-depth work on T and B lymphocyte signaling and activation.

Freshly purified human PBMCs were cultured for 4 days in the absence versus presence of serial dilutions of test products. Three parallel sets of cultures were established, where one tested the direct effect of test product on lymphocyte proliferation, and the two others examined the possible interference with response to the mitogen PHA or PWM. PHA produces a cleaner signal as it strictly induces proliferation of T lymphocytes, but PWM represents a more physiological signal mimicking the cellular interactions between monocytes/macrophages and T and B lymphocytes that occur in lymphoid tissue; therefore it is beneficial to test both in parallel. Positive controls included cells treated only with a mitogen in the absence of test product. No direct effects of product on lymphocyte proliferation were observed (data not shown). In the presence of PHA (Fig. 1A) and PWM (Fig. 1B), statistically significant decreases in proliferation of 30% and 15%, respectively, were seen with all three dilutions of NEM-AQ, indicating that pretreatment of PBMCs with NEM-AQ altered their response to subsequent signals.

Changes in Th1/Th2 cytokine levels

Supernatants were collected from 4-day cultures where PBMCs were exposed to test products in the absence versus presence of the mitogens PHA and PWM and analyzed for

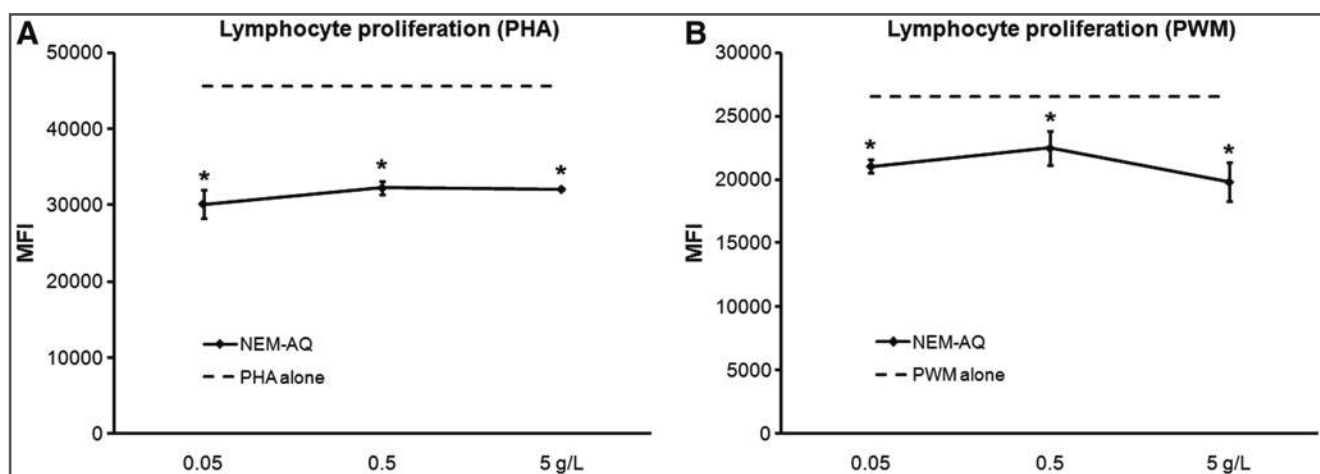


FIG. 1. Effect of aqueous extract of Natural Eggshell Membrane (NEM) (NEM-AQ) on lymphocyte proliferation in the presence of (A) phytohemagglutinin (PHA) and (B) pokeweed mitogen (PWM). Statistically significant decreases in the proliferative response of 4-day lymphocyte cultures to (A) PHA and (B) PWM were seen when cells were exposed to serial dilutions of NEM-AQ. Statistically significant differences are indicated (**P* < .05). Conditions were assayed in triplicate, and the results shown are mean \pm SD values from a representative of three separate experiments using cells from three different donors. MFI, mean fluorescence intensity.

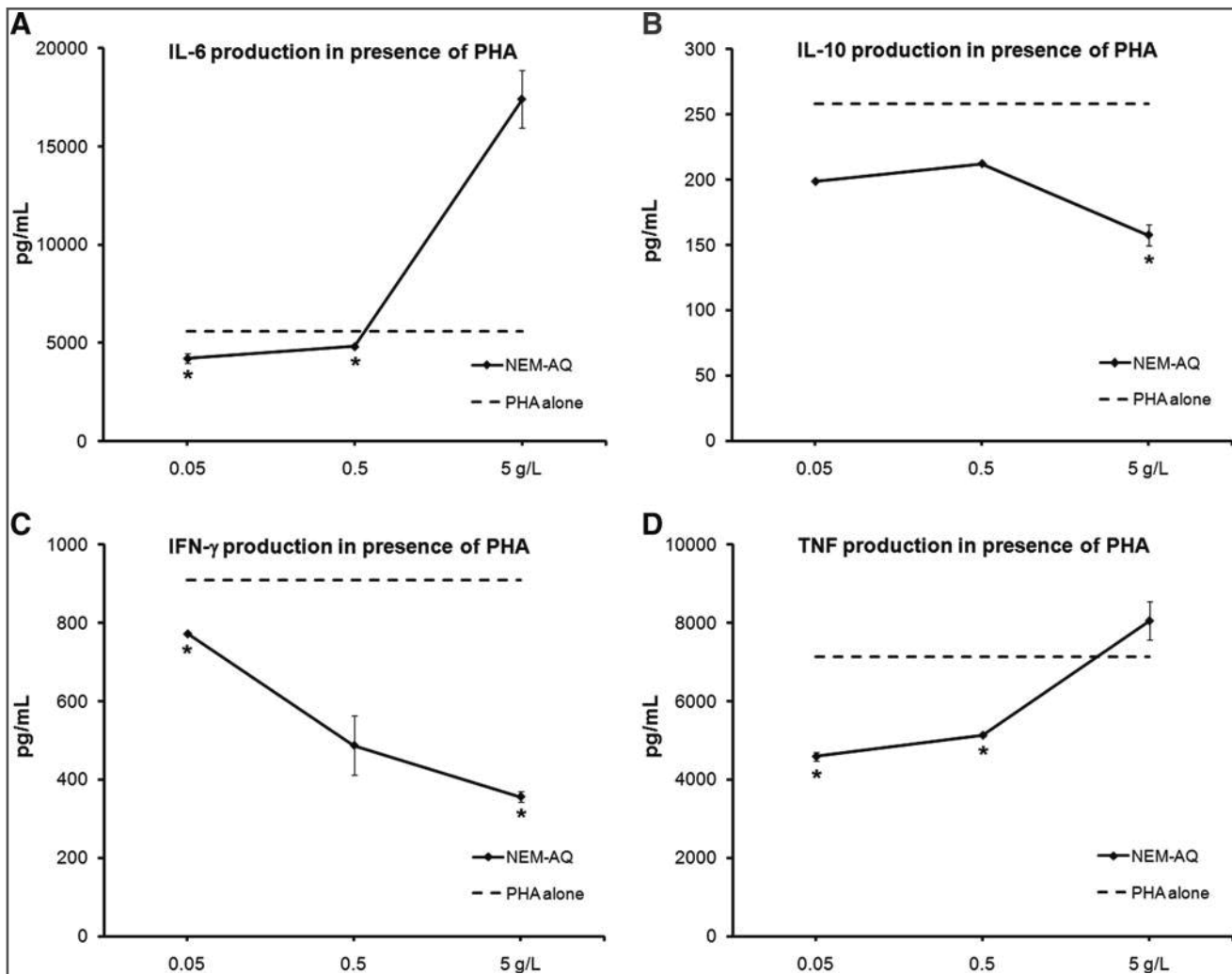


FIG. 2. Effects of NEM-AQ on production of the cytokines (A) interleukin (IL)-6, (B) IL-10, (C) interferon- γ (IFN- γ), and (D) tumor necrosis factor- α (TNF- α) by 4-day peripheral blood mononuclear cell cultures simultaneously exposed to PHA. Supernatants from 4-day peripheral blood mononuclear cell cultures were simultaneously assayed for the presence of the cytokines IL-6, IL-10, IFN- γ , and TNF- α using a flow cytometry-based assay. (A) IL-6 production in cultures exposed to serial dilutions of NEM-AQ showed a strong increase at the highest dose tested (5 g/L) and a decrease at the two lowest concentrations of NEM-AQ tested. (B) Decreases in IL-10 production were seen with all three doses of NEM-AQ in the presence of PHA. (C) The cytokine IFN- γ levels decreased in a dose-dependent manner. This reduction in IFN- γ production was over 60% at the highest concentration of NEM-AQ tested. (D) TNF- α production decreased in cultures exposed to serial dilutions of NEM-AQ. Statistically significant differences are indicated (* $P < .05$). The results shown are mean \pm SD values from a representative of three separate lymphocyte proliferation cultures using cells from three different donors.

the panel of Th1/Th2 cytokines IL-2, IL-4, IL-6, IL-10, IFN- γ , and TNF- α , using a CBA for flow cytometry. No statistically significant changes in cytokine production occurred in unstimulated cultures, whereas statistically significant changes in the cytokines IL-6, IL-10, IFN- γ , and TNF- α occurred in cultures that contained PHA (Fig. 2) or PWM (Fig. 3).

Cultures exposed to serial dilutions of NEM-AQ showed a biphasic response, including a strong increase of IL-6 at the highest dose tested (5 g/L) in the presence of both PHA and PWM, a decrease at the lower concentrations for PHA, and no effect at lower doses for PWM. Levels of IL-10, IFN- γ , and TNF- α decreased in the presence of all three doses of NEM-AQ in the presence of PHA. Effects in the presence of

PWM were only seen at higher doses. The reduction in IFN- γ production in the presence of PHA was over 60% at the highest concentration of NEM-AQ tested. At the 0.05 g/L dose, a 35% reduction in TNF- α levels was seen, indicating a strong anti-inflammatory effect of NEM-AQ on TNF- α production at lower concentrations in the presence of the T-cell mitogen PHA.

In vitro digest

An *in vitro* digest as outlined in Figure 4 was performed on NEM-AQ as well as a saline control (PBS-IVD), and the resulting material was subjected to SDS-PAGE and silver stain detection in parallel with NEM-AQ. This analysis

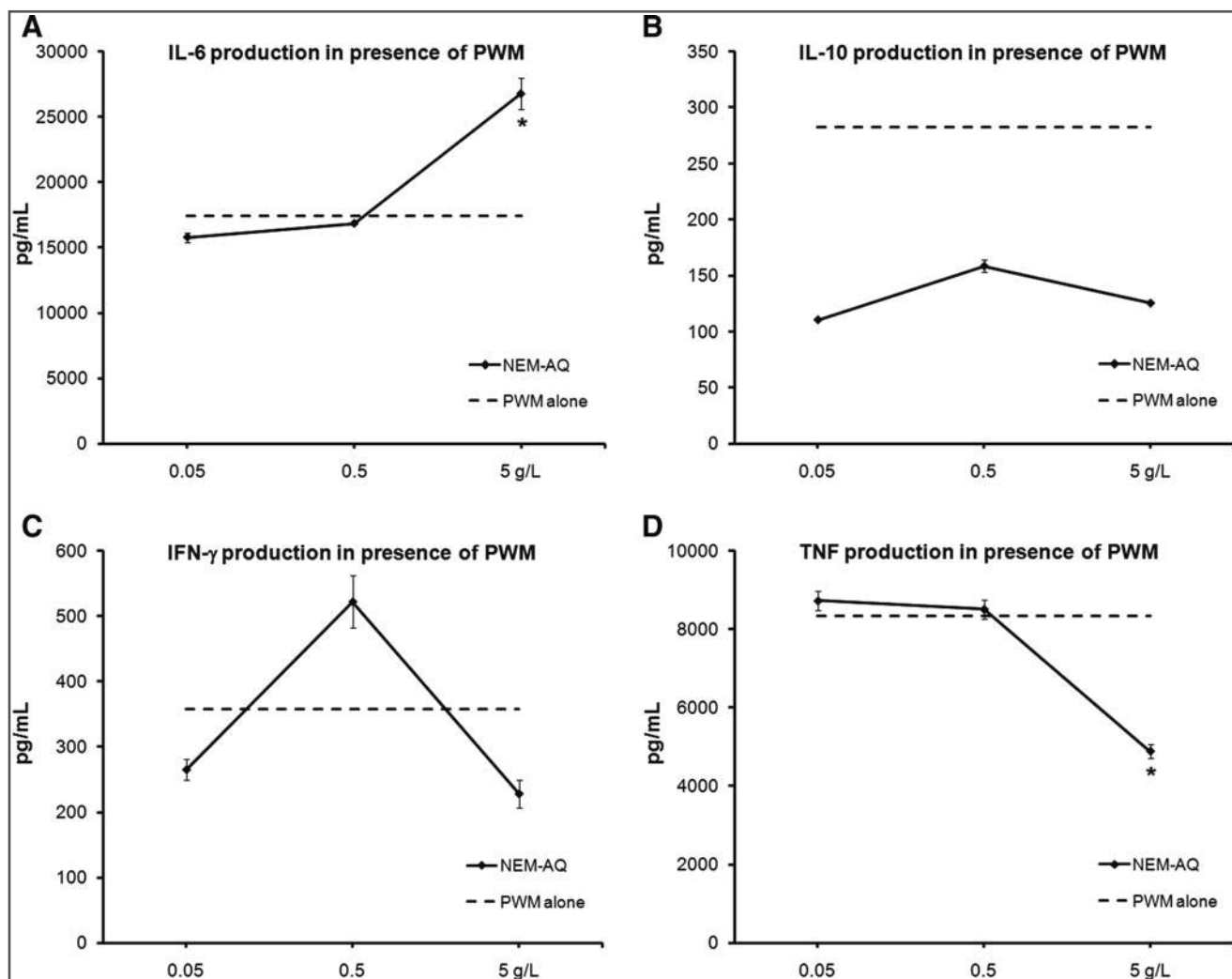


FIG. 3. Effects of NEM-AQ on production of the cytokines (A) IL-6, (B) IL-10, (C) IFN- γ , and (D) TNF- α by 4-day peripheral blood mononuclear cell cultures simultaneously exposed to PWM. Supernatants from 4-day peripheral blood mononuclear cell cultures were simultaneously assayed for the presence of cytokines using a flow cytometry-based assay. (A) IL-6 production in cultures exposed to serial dilutions of NEM-AQ showed a strong increase at the highest dose tested (5 g/L), whereas lower concentrations of NEM-AQ had no effect compared with IL-6 production in cultures exposed to PWM alone. (B) All three concentrations of NEM-AQ decreased IL-10 production in the presence of PWM about twofold. (C) Production of IFN- γ by 4-day cultures exposed to serial dilutions of NEM-AQ in the presence of PWM was affected differently depending on the concentration of NEM-AQ. These changes were not statistically significant. (D) In the presence of PWM, TNF- α production by 4-day cultures was decreased by the highest concentration of NEM-AQ, whereas lower concentrations of NEM-AQ had no effect on TNF- α production. Statistically significant differences are indicated (* $P < .05$). The results shown are mean \pm SD values from a representative of three separate lymphocyte proliferation cultures using cells from three different donors.

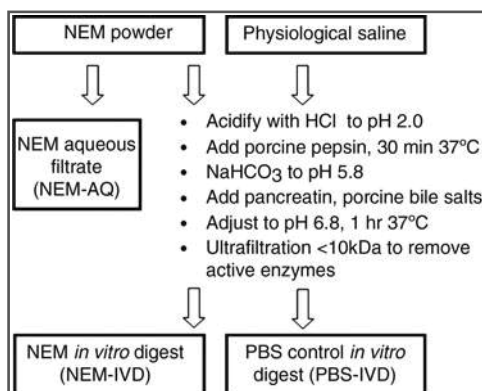


FIG. 4. Diagram outlining the *in vitro* digestion procedure. Based on methods published in the literature,^{11–13} a stepwise process was performed that incorporated digestive enzymes derived from pig (porcine) and pH adjustments in order to mimic the digestive processes occurring in the stomach and small intestine. The final digested product was returned to physiological pH and subjected to size-exclusion centrifugation using a 10-kDa filtration column in order to remove the porcine enzymes. This process was performed with NEM-AQ, resulting in the product referred to as *in vitro*-digested NEM (NEM-IVD), as well as with phosphate-buffered saline (PBS), resulting in the product referred to as PBS-IVD.

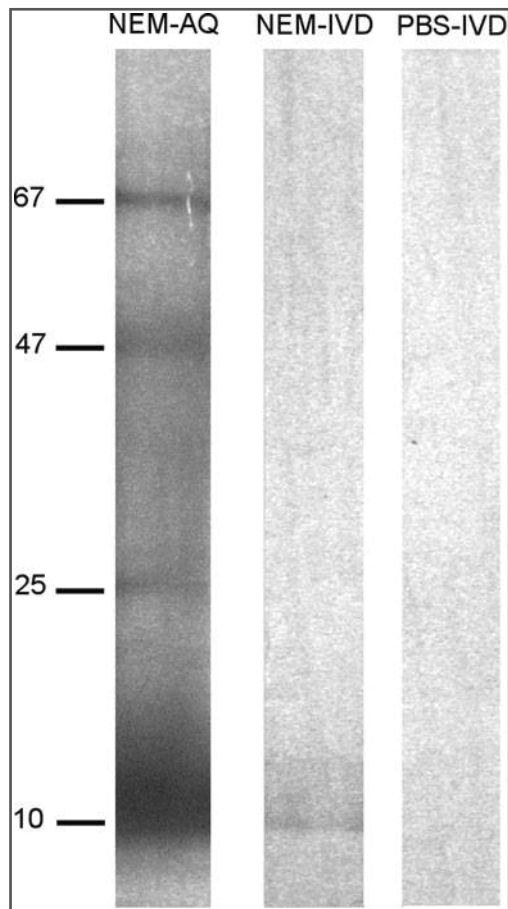


FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation of NEM-AQ, NEM-IVD and PBS-IVD. Samples were separated on a 4–15% polyacrylamide gel using denaturing conditions, and bands were visualized by silver stain. Molecular sizes of major bands in the NEM-AQ lane are indicated in kDa on the left-hand side. This presentation of the data is used to show the effects of the *in vitro* digestion of NEM on the molecular weights of the resulting digested protein products. This comparison shows a loss of higher-molecular-weight products when comparing NEM-IVD with the undigested NEM-AQ. Within the sensitivity of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stain methods, no residual enzymes or breakdown products from the *in vitro* digestion process were detectable in the PBS-IVD sample.

showed a reduction of high-molecular-weight material in the NEM-IVD sample (Fig. 5) compared with the NEM-AQ sample, whereas the PBS-IVD sample did not show any detectable protein.

Suppression of TNF- α production resulting from treatment of PBMCs with NEM-IVD

In the context of the mitogen PWM, reflecting an *in vitro* model of the cellular collaborations in lymphoid tissue, the native NEM-AQ showed an anti-inflammatory effect only at the highest dose used, and the effect returned to baseline at the lower doses. In contrast, NEM-IVD showed significant anti-inflammatory properties across a wide dose range with

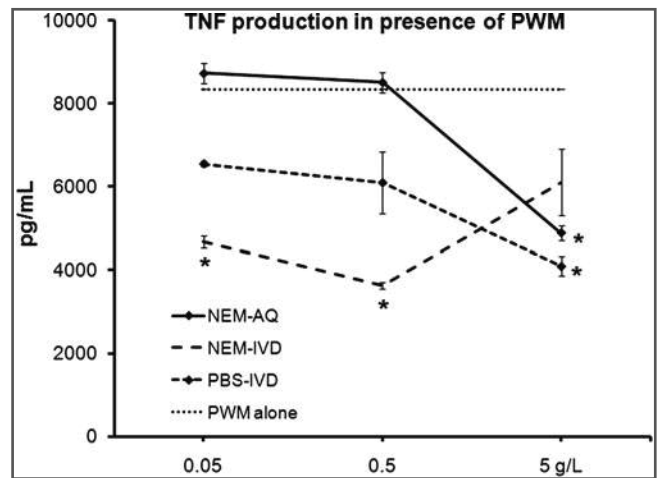


FIG. 6. Comparison of the effect of NEM-AQ, NEM-IVD, and PBS-IVD on production of the cytokine TNF- α by 4-day peripheral blood mononuclear cell cultures simultaneously exposed to PWM. TNF- α production by cultures exposed to PWM was most affected by the NEM-IVD extract. At the highest dose of extracts tested (5 g/L), all three products produced a similar reduction in TNF- α production of 30–40%. However, it can be argued that this high dose is beyond a physiological relevant dose after consumption. Therefore, the data for the two lower doses may be more relevant for predicting *in vivo* outcomes. At the two lowest doses of extracts tested, a different picture emerged. Here NEM-AQ did not affect TNF- α production, showing cytokine levels similar to baseline (cultures exposed to PWM alone). The PBS-IVD extract showed some activity, lowering TNF- α production. However, NEM-IVD had the greatest effect in lowering TNF- α production. At the 0.5 and 0.05 g/L concentrations, NEM-IVD lowered TNF- α production by 55% and 45%, respectively. Statistically significant differences are indicated (* $P < .05$). The results shown are mean \pm SD values from a representative of three separate lymphocyte proliferation cultures using cells from three different donors.

respect to TNF- α production (Fig. 6). The *in vitro*-digested saline control (PBS-IVD) had some effects on PBMC culture proliferation and cytokine production that were different from those of saline alone, suggesting the presence of residual material derived from the *in vitro* digestion process. The effects seen at the two lower doses may be most relevant for predicting *in vivo* outcomes. The data showed that in the presence of PWM, NEM-AQ had only a minor effect on TNF- α production, whereas NEM-IVD showed 45–55% suppression of TNF- α production ($P < .04$).

DISCUSSION

Osteoarthritis is often considered a local problem centered on the specific target area where bone and joint degradation is seen, such as, for example, a knee. However, osteoarthritis is a systemic disease, involving immune dysregulation and altered cytokine profile (Fig. 7). In particular, T cells likely play an important role in the pathogenesis and progression of osteoarthritis. Osteoarthritis involves infiltrating monocytes producing TNF- α .¹⁵ It is also believed that peripheral blood leukocytes, which travel through the tissues of inflamed joints, are activated

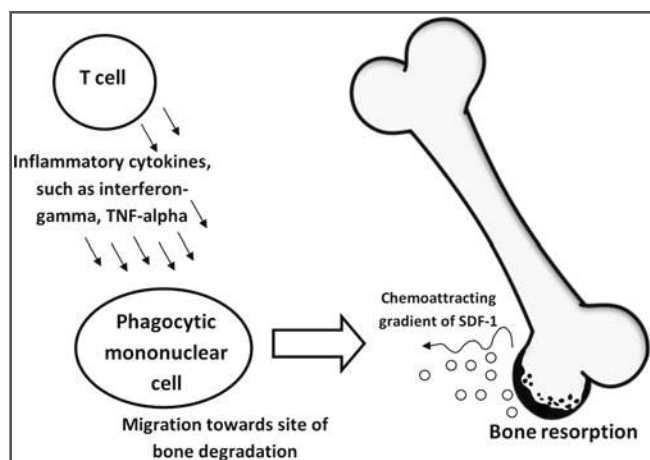


FIG. 7. Diagram showing arthritic mechanisms of action involving T-cell-derived inflammatory cytokines in activation and migration of phagocytic mononuclear cells into areas of bone destruction. SDF-1, stromal cell-derived factor-1.

through exposure to locally produced mediators of inflammation (*i.e.*, IL-1 β , TNF- α , etc.).¹⁶ We were therefore interested in studying cytokine production in peripheral blood mixed cultures including monocytes. This was accomplished through an extended proliferation/cytokine assay, where both digested and undigested NEM preparations with appropriate controls were tested in serial dilutions in the presence and absence of mitogens. Two mitogens were tested in parallel: PHA, which is a T-cell mitogen that will induce T-cell proliferation, and PWM, which is a mitogen that requires the collaboration of T cells, B cells, and monocytes in the culture.

The significance of the data must be interpreted in light of the specificity of the culture conditions in the presence of the two separate mitogens, PHA and PWM, as well as the importance of the sequence in which stimulating agents were added. PWM is an aqueous extract from *Phytolacca americana* (pokeweed) that has mitogenic properties that involve mechanisms closely mimicking events in lymph nodes and other immune tissue where antigen presentation leads to co-stimulation and collaboration of multiple cell subsets. The mechanisms involve leukocyte aggregation.¹⁷ RNA synthesis precedes DNA synthesis by 24 hours, after which cell division begins, involving up to 60% of the peripheral blood lymphocyte fraction.¹⁸ The activation process involves T lymphocytes, B lymphocytes, and phagocytic mononuclear cells¹⁹ in tandem and generates both T cell- and B cell-derived cytokines,²⁰ leading to generation of immunoglobulin-secreting plasma cells²¹ and a shift in CD45 isoform expression indicative of plasma cell differentiation.²² In contrast, an extract from *Phaseolus vulgaris* (red kidney bean) called PHA predominantly activates T lymphocytes, even though some B-cell activation can be seen as a result of the activated T cells triggering some B lymphocytes into proliferation.²³ Therefore, these two mitogens were used as a method to shed light on events that are strictly T cell mediated versus

events that require complex cellular collaboration (B cells and T cells).

The reduction of proliferation in NEM-treated cultures should not be seen as a suppression of a mitogenic response, but rather as evidence that NEM has leukocyte signaling properties of its own. The sequential addition of NEM first, followed by mitogens after 5 minutes, allowed compounds in NEM to engage signaling in target cells so when the mitogens were subsequently added the resultant signal was diminished.

Because NEM affected both PHA and PWM mitogenicity, but in different ways, this finding suggests specific mechanisms, including that NEM contains compounds directly able to modulate T-cell activation, and that NEM also has immune-modulating properties in the context of a more physiological activation process, such as in the PWM model of lymphocyte activation.

Cytokine production was affected in the cultures, with significant changes in three inflammatory cytokines: IL-6, IFN- γ , and TNF- α . It is interesting that this did not change when NEM was passed through the *in vitro* digestion protocol, except for TNF- α . The reduction in TNF- α production with NEM-IVD was seen at 100-fold lower doses than with undigested NEM. In the case of PWM stimulation, NEM-IVD showed anti-inflammatory properties by drastically reducing the production of TNF- α , in contrast to the mild increase in TNF- α production when cells were pretreated with NEM-AQ. Thus, the *in vitro* digestion potentiated the anti-inflammatory action of NEM, so that much lower doses of NEM-IVD were seen to produce similar effects as 25-fold higher doses of NEM-AQ. This is relevant for suggesting anti-inflammatory mechanisms *in vivo* after consumption of NEM and subsequent digestion in the stomach.

The dose-responses seen in the different assays were in several cases nonlinear. This may be attributed to several confounding factors associated with the highest dose, suggesting that the biological effects observed at lower doses should receive the most attention. It may be argued that the highest dose we used (5 g/L) exceeds a likely physiologically relevant dose. However, it may also be argued that this dose may be reached locally along the intestinal mucosa after consumption. We suggest that the highest dose (5 g/L) is quite high and that the biological responses seen at the two lower doses may be more relevant for predicting *in vivo* outcomes. There is also the possibility that calcium may have been an issue at the highest dose. NEM contains some calcium from unseparated eggshell, so for the most part this will be insoluble calcium carbonate. At the 5 g/L dose of NEM, the amount of calcium present may still be sufficient to interfere with cellular signaling. Further dilution of NEM may dilute calcium to insignificant levels. As it is therefore unlikely that calcium contributed to the different responses seen at the lowest dose, it cannot be completely ruled out as a potential mechanism of disturbing or abrogating cellular signaling at the highest dose used.

In the case of the TNF- α cytokine testing, the most interesting difference between the effects of NEM-AQ and NEM-IVD was the reduction in TNF- α production in the

presence of PWM that occurred with all three concentrations of NEM-IVD. These reductions in TNF- α production were strongest with the lowest doses of NEM-IVD, and this effect was opposite to that seen for NEM-AQ. Although the *in vitro* digestion procedure introduced compounds that were not completely removed by the size exclusion centrifugation step and that possessed bioactivity, the digestion process nevertheless increased the ability of NEM-AQ to reduce TNF- α production in 4-day PBMC cultures in the presence of PWM. The effect of NEM-IVD on TNF- α production in the presence of PWM was also different from the effect resulting from treatment of PBMC cultures with PBS-IVD and points to activities unique to the NEM-IVD product.

In particular, the result of NEM-AQ and NEM-IVD reducing TNF- α production is of interest in terms of identifying mechanisms of action pertaining to arthritis conditions because this cytokine is known to attract cell infiltration into arthritic joints and contribute to the inflammation within the joint.

Comparison of the effects of NEM-AQ and NEM-IVD on cytokine production by PBMC cultures revealed some differences that could not be entirely attributed to activities derived from the *in vitro* digestion process itself (such as enzyme breakdown products or residual bile salts that were not removed by the size exclusion centrifugation step). This unique effect of NEM-IVD with respect to TNF- α clearly warrants efforts to further investigate the effects of *in vitro* digestion on NEM. This is of particular importance as several biological TNF- α -inhibiting drugs have proven quite effective in treating arthritis but have been shown to have infrequent but often severe side effects.²⁴ A treatment, such as NEM, that has immunomodulatory properties that are likely more diffuse could potentially avoid the unfortunate side effects of the currently available biological drugs.

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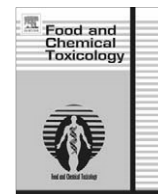
AUTHOR DISCLOSURE STATEMENT

K.F.B. and G.S.J. are employed by NIS Labs, an independent contract research laboratory. K.J.R. is employed by ESM Technologies, LLC in the function of Director of Scientific and Regulatory Affairs. The authors have no other financial interest in the subject matter.

REFERENCES

- Gonzalez EA: The role of cytokines in skeletal remodeling: possible consequences for renal osteodystrophy. *Nephrol Dial Transplant* 2000;15:945–950.
- Komano Y, Nanki T, Hayashida K, Taniguchi K, Miyasaka N: Identification of a human peripheral blood monocyte subset that differentiates into osteoclasts. *Arthritis Res Ther* 2006;8:R152.
- Tsutsumi H, Tanaka T, Ohashi N, Masuno H, Tamamura H, Hiramatsu K, Araki T, Ueda S, Oishi S, Fujii N: Therapeutic potential of the chemokine receptor CXCR4 antagonists as multifunctional agents. *Biopolymers* 2007;88:279–289.
- Santiago B, Baleux F, Palao G, Gutierrez-Canas I, Ramirez JC, Arenzana-Seisdedos F, Pablos JL: CXCL12 [SDF-1] is displayed by rheumatoid endothelial cells through its basic amino-terminal motif on heparan sulfate proteoglycans. *Arthritis Res Ther* 2006;8:R43.
- Ruff KJ, Devore DP, Leu MD, Robinson MA: Eggshell membrane: a possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin Interv Aging* 2009;4:235–240.
- Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW: Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin Rheumatol* 2009;28:907–914.
- Wong M, Hendrix MJC, von der Mark K, Little C, Stern R: Collagen in the egg shell membranes of the hen. *Dev Biol* 1984;104:28–36.
- Baker JR, Balch DA: A study of the organic material of hen's-egg shell. *Biochem J* 1962;82:352–361.
- Long FD, Adams RG, DeVore DP, inventors; New Life Resources, LLC, assignee: Preparation of hyaluronic acid from eggshell membrane. U.S. Patent 6,946,551. September 20, 2005.
- Picard J, Paul-Gardais A, Vedel M: Sulfated glycoproteins from egg shell membranes and hen oviduct. Isolation and characterization of sulfated glycopeptides. *Biochim Biophys Acta* 1973;320:427–441.
- Yun S, Habicht J-P, Miller DD, Glahn RP: An *in vitro* digestion/Caco-2 cell culture system accurately predicts the effects of ascorbic acid and polyphenolic compounds on iron bioavailability in humans. *J Nutr* 2004;134:2717–2721.
- Boyer J, Brown D, Liu RH: *In vitro* digestion and lactase treatment influence uptake of quercetin and quercetin glucoside by the Caco-2 cell monolayer. *Nutr J* 2005;4:1–15.
- Toor RK, Savage GP, Lister CE: Release of antioxidant components from tomatoes determined by an *in vitro* digestion method. *Int J Food Sci Nutr* 2009;60:119–129.
- Jensen GS, Benson KF, Carter SG, Endres JR: GanedenBC30 cell wall and metabolites: anti-inflammatory and immune modulation effects in vitro. *BMC Immunol* 2010;11:15.
- Sakkas L, Platsoucas C: The role of T cells in the pathogenesis of osteoarthritis. *Arthritis Rheum* 2007;56:409–424.
- Attur M, Belitskaya-Lévy I, Oh C, Krasnokutsky S, Greenberg J, Samuels J, Smiles S, Lee S, Patel J, Al-Mussawir H, McDaniel G, Kraus VB, Abramson SB: Increased interleukin-1 β gene expression in peripheral blood leukocytes is associated with increased pain and predicts risk for progression of symptomatic knee osteoarthritis. *Arthritis Rheum* 2011;63:1908–1917.
- Börjeson J, Reisfeld R, Chessin LN, Welsh PD, Douglas SD: Studies on human peripheral blood lymphocytes *in vitro*. I. Biological and physicochemical properties of the pokeweed mitogen. *J Exp Med* 1966;124:859–872.
- Chessin LN, Börjeson J, Welsh PD, Douglas SD, Cooper HL: Studies on human peripheral blood lymphocytes *in vitro*. II. Morphological and biochemical studies on the transformation of lymphocytes by pokeweed mitogen. *J Exp Med* 1966;124:873–884.

19. de Vries E, Lafeber GJ, van der Weij JP, van Buijsen AC, Leijh PC, Cats A: Pokeweed-mitogen induced lymphocyte proliferation: the effect of stimulation on mononuclear phagocytic cells. *Immunology* 1980;40:177–182.
20. Weksler ME, Kuntz MM: Synergy between human T and B lymphocytes in their response to phytohaemagglutinin and pokeweed mitogen. *Immunology* 1976;31:273–281.
21. Hammarström L, Bird AG, Britton S, Smith CI: Pokeweed mitogen induced differentiation of human B cells: evaluation by a protein A haemolytic plaque assay. *Immunology* 1979;38:181–189.
22. Jensen GS, Poppema S, Mant MJ, Pilarski LM: Transition in CD45 isoform expression during differentiation of normal and abnormal B cells. *Int Immunol* 1989;1:229–236.
23. Greaves M, Janossy G, Doenhoff M: Selective triggering of human T and B lymphocytes *in vitro* by polyclonal mitogens. *J Exp Med* 1974;140:1–18.
24. Hochberg MC, Lebowitz MG, Plevy SE, Hobbs KF, Yocum DE: The benefit/risk profile of TNF-blocking agents: findings of a consensus panel. *Semin Arthritis Rheum* 2005;34: 819–836.



Safety evaluation of a natural eggshell membrane-derived product

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ABSTRACT

Natural Eggshell Membrane (NEM[®]) is a novel dietary ingredient that contains naturally occurring glycosaminoglycans and proteins essential for maintaining healthy joint and connective tissues. NEM[®] was evaluated for safety via *in vitro* and *in vivo* toxicological studies. This included testing for cytotoxicity, genotoxicity, acute oral toxicity, and 90-day repeated-dose oral toxicity. NEM[®] did not exhibit any cytotoxic effects at a dose of 100 µg in an *in vitro* human cell viability assay after incubation for up to 20 h. NEM[®] did not exhibit any genotoxic effects in an *in vitro* assay of four strains of histidine-dependent *Salmonella typhimurium* and one strain of tryptophan-dependent *Escherichia coli* at a dose of up to 5000 µg/plate. NEM[®] did not exhibit any signs of acute toxicity in rats at a single oral dose of up to 2000 mg/kg body weight, nor signs of toxicity (via urinalysis, hematology, clinical chemistry, or histopathological evaluation) in rats at a repeated oral dose of up to 2000 mg/kg body weight per day for 90 days. The results of these studies suggest that NEM[®] may be safe for human consumption.

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1. Introduction

Chicken eggs have been a staple of many cultures' diets for centuries and are well-accepted as being safe to eat. Some cultures are also known to consume the egg shells and eggshell membranes in various ways (Freedman, 1981; Long 1913). Eggshell membranes

are an abundant raw material that are a novel source for naturally occurring bioactive compounds such as glucosamine (Picard et al., 1973), chondroitin sulfate (Baker and Balch, 1962), hyaluronic acid (Long et al., 2005), collagen Type I (Wong et al., 1984), and sulfur-rich proteins (Tsai et al., 2006). In US alone, an estimated 600,000 tons of eggshells are produced annually as a by-product of the egg products industry (United Nations Food and Agricultural Organization, 2004). Disposal of these eggshells creates an environmental and financial burden and, therefore, alternative uses for these materials are of obvious benefit. Technologies have recently emerged that allow for the efficient separation of the eggshell membranes from the egg shell commercially, making possible the development of value-added products from both materials (Adams and Franklin, 2006).

Egg shells are a natural source for calcium and have been evaluated for safety in a number of animal and human studies carried out primarily in Europe and Asia (Hirasawa et al., 2001; Schaafsma and Beelen, 1999; Schaafsma and Pagan, 1999; Stancikova et al., 1996; Svik et al., 1996). Eggshell meal (both shell and membrane) has been officially recognized by the Association of American Feed Control Officials (AAFCO) as safe as a feed additive for both companion and livestock animals since 1982 (Association of American Feed Control Officials Official Publication, 2009). To our knowledge, however, eggshell membrane or its derivatives have not previously been evaluated for safety through standard *in vitro* and *in vivo* toxicological studies. To this end, NEM[®], an eggshell membrane derived product for oral administration, was evaluated for cytotoxicity,

Abbreviations: AAFCO, Association of American Feed Control Officials; ABSRET, absolute reticulocytes; A/G, albumin/globulin ratio; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BAS, basophils; BLD, occult blood; BUN, urea nitrogen; BW, body weight; CA, calcium; CFU, colony forming units; CHOL, cholesterol; CK, creatine phosphokinase; CL, chloride; CREA, creatinine; EOS, eosinophils; GGT, gamma-glutamyltransferase; GLOB, globulin; GLU, glucose; HCT, hematocrit; HED, human equivalent dose; HGB, hemoglobin; K, potassium; KET, ketones; LDH, lactate dehydrogenase; LEU, leukocytes; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NA, sodium; NAEOL, no adverse effect observed level; NEG, negative; NEM, Natural Eggshell Membrane; NEUT, neutrophils; NIT, nitrite; NOEL, no observable effect level; OECD, Organisation for Economic Co-operation and Development; PBMC, peripheral blood mononuclear cells; PHOS, inorganic phosphorus; PLAT, platelets; PT, prothrombin time; RBC, red blood cell count; SG, specific gravity; TBILL, total bilirubin; TPRO, total protein; TRIG, triglycerides; UBILL, urinary bilirubin; UGLU, urinary glucose; UPRO, urinary protein; URO, urobilinogen; VOL, volume; WBC, white blood cell count.

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genotoxicity, acute oral toxicity, and 90-day repeated dose oral toxicity. The results of these studies are presented herein.

2. Materials and methods

2.1. Preparation and storage of NEM®

ESM Technologies, LLC (Carthage, MO, USA) has developed a “green” manufacturing process to efficiently and effectively separate eggshell membrane from eggshells on a commercial scale to create an essentially shell-free eggshell membrane (Adams and Franklin, 2006). The isolated membrane is then partially hydrolyzed in an aqueous medium using a proprietary process and dry-blended to produce Natural Eggshell Membrane (NEM®) powder. Compositional analysis of NEM® conducted by ESM has identified a high content of protein and moderate quantities of glucosamine, chondroitin sulfate, hyaluronic acid, and collagen. The composition of NEM® has been found to be quite consistent between different manufacturing batches, as well as with differing sources of eggs (i.e. White Leghorn versus Rhode Island Red chickens). Real-time stability studies have demonstrated that NEM® can be stored under ambient conditions for later use for up to 3 years from the date of manufacture.

2.2. Cytotoxicity evaluation

Cytotoxicity testing was performed by Consumer Product Testing Company (Fairfield, NJ USA). Human-derived epidermal keratinocytes (EpiDerm™ *in vitro* cytotoxicity system, MatTek Corp., Ashland, MA USA) were incubated with either distilled water (negative control), 100 µg of NEM® in 100 µL of distilled water, or 100 µL of 1% Triton X-100 (positive control) at 37 °C (5% carbon dioxide and ≥90% humidity) for 1, 4.5, and 20 h. Following the incubation period, the samples were evaluated for keratinocyte viability. Cell viability was determined through the use of a yellow water-soluble tetrazolium salt (MTT) that is reduced to a purple

Table 1

Percent of viable cells in NEM® and Triton X-100 (positive control) treated samples after various incubation periods in the EpiDerm™ *in vitro* cytotoxicity system.

Incubation period (h)	NEM®	Triton X-100
1	122	79
4.5	104	27
20	101	6

formazan derivative by succinate dehydrogenase in the mitochondria of viable cells. Substances that damage this mitochondrial enzyme inhibit the reduction of MTT. Therefore the amount of MTT reduced in a cell culture is proportional to the number of viable cells. A Dynatech MR 4000 Automatic Microplate Reader (Dynatech Laboratories, Inc., Alexandria, VA USA) was used to determine the absorbance of UV light in each sample at 570 nm. The absorbance of the negative control was defined as 100% viability for test article and positive control evaluation.

2.3. Mutagenicity evaluation

Mutagenicity (Ames Reverse Mutation test) testing was performed by Pharmaceutical Control and Development Laboratory Co. Ltd. (Budapest, Hungary) according to the OECD Guideline for Testing of Chemicals (Guideline No. 471, adopted 21 July 1997). A preliminary cytotoxicity assessment was performed at 5, 10, 50, 100, 500, 1000, and 5000 µg/plate to determine the appropriate dose range for mutagenicity evaluation. As no significant cytotoxic effect was observed, the five highest doses were then used in the subsequent mutagenicity evaluation. To evaluate mutagenicity, four strains of histidine-dependent *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and one strain of tryptophan-dependent *Escherichia coli* (WP2) (Xenometrix GmbH, Switzerland) were tested in triplicate at the five highest doses (50, 100, 500, 1000, and 5000 µg/plate) of NEM® in both the presence

Table 2

Revertant colonies per plate of histidine-dependent *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and tryptophan-dependent *Escherichia coli* (WP2) in control (0 µg/plate) and NEM® dosed plates (50–5000 µg/plate), both in the presence and absence of Aroclor™ 1254-induced rat liver S9 metabolic activation system in the Ames Reverse Mutation Test.

Dose (µg/plate)	Bacterial strains evaluated									
	No activation					With S9 activation				
	TA98	TA100	TA1535	TA1537	WP2	TA98	TA100	TA1535	TA1537	WP2
0	31 ± 3	176 ± 11	25 ± 3	9 ± 2	87 ± 9	42 ± 5	178 ± 14	23 ± 2	8 ± 3	97 ± 6
50	35 ± 3	181 ± 9	26 ± 3	10 ± 2	85 ± 6	39 ± 5	179 ± 7	22 ± 4	9 ± 3	96 ± 7
100	32 ± 3	179 ± 12	28 ± 6	9 ± 2	87 ± 14	43 ± 3	179 ± 8	21 ± 2	7 ± 3	95 ± 17
500	33 ± 5	176 ± 11	22 ± 3	12 ± 1	94 ± 12	39 ± 3	177 ± 15	22 ± 4	8 ± 2	96 ± 9
1000	33 ± 2	181 ± 11	23 ± 3	11 ± 3	86 ± 9	40 ± 7	179 ± 8	21 ± 1	10 ± 1	95 ± 12
5000	32 ± 3	177 ± 5	26 ± 4	10 ± 1	90 ± 10	40 ± 4	178 ± 11	20 ± 4	10 ± 2	98 ± 8

Values represent means ± standard deviations.

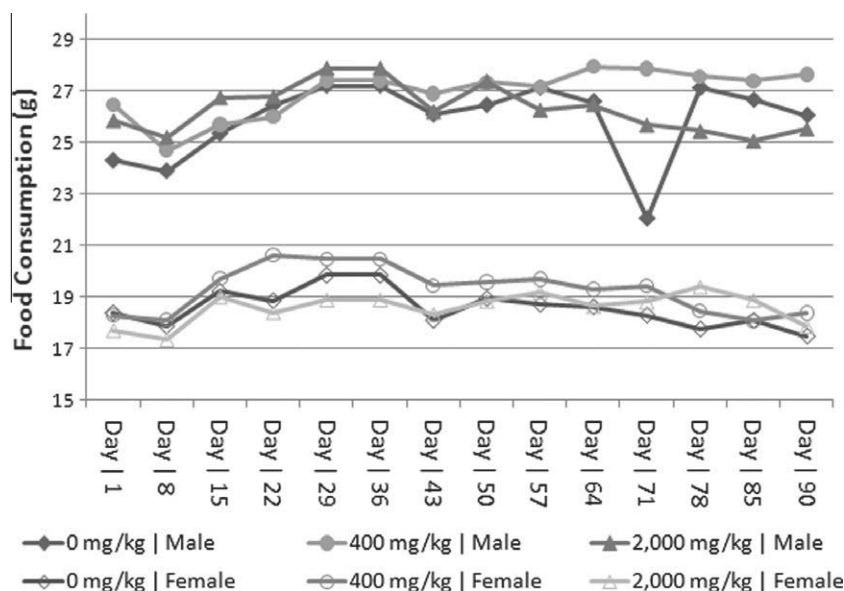


Fig. 1. Mean daily food consumption during 90-day oral toxicological evaluation of NEM® (0, 400, and 2000 mg/kg bw/day) in rats.

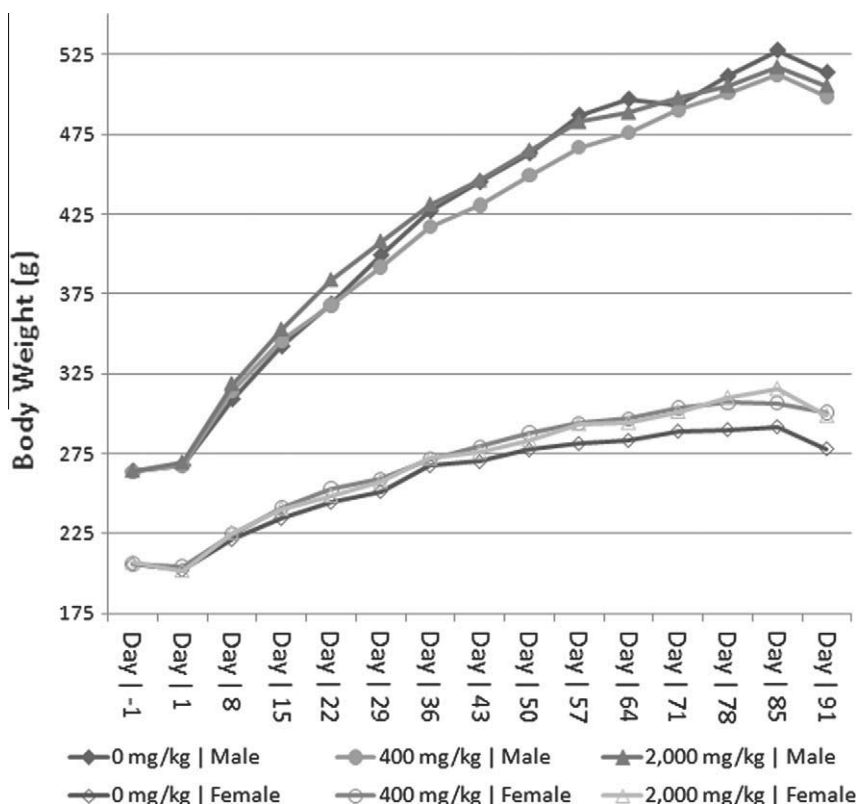


Fig. 2. Average body weight during 90-day oral toxicological evaluation of NEM® (0, 400, and 2000 mg/kg bw/day) in rats.

and absence of Aroclor™ 1254-induced rat liver S9 metabolic activation system (Trinova Biochem GmbH, Germany). Both positive controls (9-aminoacridine, 2-aminoanthracene, benzo- α -pyrene, methyl methanesulfonate, 2-nitrofluorene, and sodium azide) with and without S9 activator and negative controls (sterile distilled water) with and without S9 activator were included in the evaluation. This was done to ensure the test system was functioning properly (positive controls) and to obtain baseline revertant frequencies for the various strains of bacteria used in the study (negative controls). The plates were counted after 72 h of incubation at 37 °C. Results of the initial assay were confirmed with a repeated assay using a 2.5× increase in S9 activator.

2.4. Animal models of toxicology

2.4.1. Acute oral toxicity in rats

Testing was performed by Pharmaceutical Control and Development Laboratory Co. Ltd. (Budapest, Hungary) following U.S. Food and Drug Administration, Title 21, Code of Federal Regulations, Part 58: Good Laboratory Practices (GLP) Regulations for Nonclinical Laboratory Studies (21 CFR 58). No more than 20 min prior to use, NEM® powder was suspended in 1% methylcellulose in distilled water at a concentration of 100 mg/mL and 200 mg/mL, corresponding to a dose volume of 10 mL/kg. After a 16-h fasting period, the NEM® suspension was administered as a single dose at either 1000 or 2000 mg/kg body weight (bw) by oral gavage to 6-week-old Sprague–Dawley (CrI:CD) rats (Charles River Laboratories Hungary Ltd., Isaszeg, Hungary) in groups of 10 (five animals per sex per group, randomized by weight within an interval of $\pm 20\%$ from the mean, M: 199 ± 5.6 g bw, F: 147 ± 7.3 g bw). The rats were acclimatized for 5 days prior to study commencement and were housed individually in cages under standard experimental conditions (22 ± 3 °C; 30–70% humidity; 12-h light/dark cycle) and had access to standard rat chow (Sniff SM R/M – Z + H, Sniff Spezialdiäten GmbH, Germany) and water *ad libitum*. The rats were observed daily for 14 days following administration of the test article for mortality and clinical signs of toxicity (changes in gait, posture, skin, fur, eyes, or mucous membranes; occurrence of diarrhea, lacrimation, unusual respiratory pattern, somnolence, clonic or tonic movements, etc.). On day 15, all animals were euthanized by hyperanesthesia and underwent gross pathological examination for signs of toxicity via necropsy.

2.4.2. 90-day oral toxicity in rats

Testing was performed by Ricerca Biosciences, LLC (Concord, OH, USA) following U.S. Food and Drug Administration, Title 21, Code of Federal Regulations, Part 58: Good Laboratory Practices (GLP) Regulations for Nonclinical Laboratory Studies (21 CFR 58). The test article was prepared weekly by suspending NEM® powder in

0.5% methylcellulose in distilled water at a concentration of 40 and 200 mg/mL, corresponding to a dose volume of 10 mL/kg. The test article was stored at approximately 4 °C with constant stirring between daily uses. The NEM® suspension was administered daily at doses of 0 (control, vehicle only), 400, or 2000 mg/kg bw/day by oral gavage to 9-week-old Sprague–Dawley (CrI:CD) rats (Charles River Laboratories Inc., Portage, MI, USA) in groups of 10 (five animals per sex per group, randomized by weight within an interval of $\pm 6\%$ from the mean, M: 264.2 ± 8.9 g bw, F: 206.6 ± 7.7 g bw) for 90 consecutive days. The rats were acclimatized for 7 days prior to study commencement and were housed individually in cages under standard experimental conditions (22 ± 3 °C; 30–70% humidity; 12-h light/dark cycle; minimum 10 room air changes per hour) and had access to standard rat chow (Teklad Global Diet 2016, Harlan Laboratories, Indianapolis, IN, USA) and water *ad libitum*. The rats were observed twice daily (at least 6 h apart) following administration of the test article for mortality and clinical signs of toxicity (described previously) during the 90-day study period.

The animals were placed in metabolism caging overnight and urine excreted by each animal was collected on day 91 for urinalysis. The parameters for urinalysis included: volume, color, clarity, specific gravity, pH, occult blood, protein, leukocytes, bilirubin, ketones, glucose, nitrite, and urobilinogen. On day 91, all animals were euthanized by hyperanesthesia and underwent gross pathological examination for signs of toxicity via necropsy. All organs, mucosa, body cavities, etc. were examined for gross pathological changes. Major organs and major endocrine glands (pituitary, adrenal, thymus, thyroid, sex, etc.) were weighed and organ/body weight ratios and organ/brain weight ratios were calculated. Tissue samples from select organs (adrenal glands, brain, heart, kidneys, liver, lungs, pancreas, and spleen) from the control and 2000 mg/kg test animals were preserved, fixed, and stained (hematoxylin and eosin) for histopathological evaluation via light microscopy. Using whole blood, hematological and coagulation analyses were carried out. The parameters for hematological analysis included: red blood cell count (RBC), reticulocyte count (ABSRET), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) and white blood cell count (WBC) (including lymphocyte, monocyte, basophil, eosinophil, and neutrophil distribution). The parameters for coagulation analysis included: platelet count (PLAT), mean platelet volume (MPV), prothrombin time (PT), and activated partial thromboplastin time (APTT). Additionally, clinical chemistry was evaluated including sodium (NA), potassium (K), calcium (CA), chloride (CL), glucose (GLU), creatinine (CREA), total bilirubin (TBIL), urea nitrogen (BUN), total protein (TPRO), albumin (ALB), globulin (GLOB), albumin/globulin ratio (A/G), cholesterol (CHOL), triglycerides (TRIG), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), creatine phosphokinase (CK), and inorganic phosphorus (PHOS).

Table 3

Summary of organ weight data after 90-day oral administration of NEM®. Data are shown as means ± standard deviations (n = 5).

	Male			Female		
	Dose (mg/kg body weight/d)			Dose (mg/kg body weight/d)		
	0	400	2000	0	400	2000
<i>Mean organ weights (g)</i>						
Body Weight	513.6 ± 60.7	498.6 ± 56.7	505.1 ± 53.2	278.0 ± 21.2	300.8 ± 15.8	298.4 ± 10.0
Brain	2.08 ± 0.12	2.18 ± 0.11	2.09 ± 0.06	1.94 ± 0.05	1.89 ± 0.15	1.92 ± 0.08
Liver	12.77 ± 1.94	12.72 ± 1.55	13.26 ± 1.63	7.37 ± 0.90	7.87 ± 0.47	7.73 ± 0.37
Heart	1.55 ± 0.11	1.74 ± 0.09 ^a	1.61 ± 0.13	1.06 ± 0.14	1.07 ± 0.07	1.07 ± 0.18
Lungs	2.24 ± 0.36	2.49 ± 0.42	2.91 ± 0.71	1.88 ± 0.37	1.69 ± 0.20	1.95 ± 0.27
Kidneys	2.79 ± 0.15	3.08 ± 0.30	3.30 ± 0.10 ^{**}	1.90 ± 0.12	1.90 ± 0.18	1.84 ± 0.15
Spleen	0.957 ± 0.129	0.926 ± 0.182	0.925 ± 0.202	0.478 ± 0.054	0.546 ± 0.027	0.497 ± 0.065
Testes	3.51 ± 0.21	3.46 ± 0.30	3.64 ± 0.20	–	–	–
Epididymides	1.56 ± 0.16	1.72 ± 0.11	1.72 ± 0.23	–	–	–
Ovaries	–	–	–	0.102 ± 0.028	0.127 ± 0.053	0.164 ± 0.017 ^a
Uterus	–	–	–	0.767 ± 0.102	0.816 ± 0.139	0.751 ± 0.129
Adrenal glands	0.063 ± 0.015	0.056 ± 0.011	0.065 ± 0.008	0.072 ± 0.013	0.073 ± 0.004	0.080 ± 0.013
Thymus	0.352 ± 0.104	0.418 ± 0.145	0.412 ± 0.118	0.318 ± 0.051	0.337 ± 0.026	0.341 ± 0.076
<i>Mean organ weight relative to body weight (g/kg)</i>						
Brain	4.10 ± 0.52	4.41 ± 0.58	4.18 ± 0.42	7.02 ± 0.66	6.28 ± 0.44	6.43 ± 0.39
Liver	24.80 ± 1.32	25.51 ± 1.12	26.21 ± 0.58	26.46 ± 2.12	26.16 ± 0.45	25.91 ± 1.54
Heart	3.03 ± 0.19	3.53 ± 0.33	3.19 ± 0.21 ^a	3.79 ± 0.36	3.56 ± 0.20	3.58 ± 0.53
Lungs	4.39 ± 0.72	5.01 ± 0.75	5.77 ± 1.34	6.74 ± 0.94	5.64 ± 0.88	6.56 ± 1.10
Kidneys	5.48 ± 0.45	6.24 ± 0.89	6.58 ± 0.52	6.85 ± 0.56	6.33 ± 0.56	6.18 ± 0.65
Spleen	1.88 ± 0.34	1.86 ± 0.31	1.84 ± 0.42	1.72 ± 0.19	1.82 ± 0.05	1.66 ± 0.21
Testes	6.89 ± 0.62	6.99 ± 0.91	7.30 ± 1.07	–	–	–
Epididymides	3.07 ± 0.52	3.48 ± 0.28	3.47 ± 0.82	–	–	–
Ovaries	–	–	–	0.371 ± 0.110	0.425 ± 0.181	0.551 ± 0.068
Uterus	–	–	–	2.77 ± 0.40	2.70 ± 0.36	2.52 ± 0.48
Adrenal glands	0.123 ± 0.024	0.111 ± 0.020	0.130 ± 0.025	0.259 ± 0.052	0.242 ± 0.025	0.269 ± 0.042
Thymus	0.681 ± 0.173	0.821 ± 0.198	0.829 ± 0.283	1.15 ± 0.22	1.12 ± 0.09	1.14 ± 0.23
<i>Mean organ weight relative to brain weight (g/g)</i>						
Liver	6.15 ± 1.04	5.87 ± 0.89	6.34 ± 0.79	3.80 ± 0.47	4.18 ± 0.21	4.04 ± 0.22
Heart	0.743 ± 0.055	0.803 ± 0.055	0.767 ± 0.061	0.544 ± 0.068	0.568 ± 0.028	0.560 ± 0.092
Lungs	1.07 ± 0.15	1.15 ± 0.20	1.39 ± 0.32	0.972 ± 0.190	0.898 ± 0.118	1.02 ± 0.15
Kidneys	1.34 ± 0.11	1.42 ± 0.21	1.58 ± 0.06	0.977 ± 0.048	1.01 ± 0.04	0.960 ± 0.055
Spleen	0.460 ± 0.065	0.425 ± 0.072	0.443 ± 0.101	0.247 ± 0.034	0.290 ± 0.013	0.259 ± 0.030
Testes	1.69 ± 0.07	1.59 ± 0.18	1.74 ± 0.10	–	–	–
Epididymides	0.750 ± 0.106	0.794 ± 0.067	0.823 ± 0.121	–	–	–
Ovaries	–	–	–	0.053 ± 0.015	0.067 ± 0.024	0.086 ± 0.008 ^a
Uterus	–	–	–	0.396 ± 0.060	0.435 ± 0.081	0.393 ± 0.072
Adrenal glands	0.030 ± 0.007	0.026 ± 0.006	0.031 ± 0.005	0.037 ± 0.007	0.039 ± 0.005	0.042 ± 0.008
Thymus	0.170 ± 0.054	0.193 ± 0.072	0.198 ± 0.060	0.164 ± 0.025	0.180 ± 0.024	0.179 ± 0.044

– = No data.

^a $p < 0.05$.^{**} $p < 0.01$.**Table 4**

Urinalysis parameters after 90-day oral administration of NEM® (0, 400, and 2000 mg/kg bw/day) in rats. Data are shown as means ± standard deviations (n = 5).

	Male			Female		
	Dose (mg/kg bw/day)			Dose (mg/kg bw/day)		
	0	400	2000	0	400	2000
VOL (mL)	12.4 ± 5.9	14.4 ± 3.4	10.2 ± 5.9	6.2 ± 3.4	8.4 ± 3.2	9.8 ± 5.5
pH	6.5 ± 0.4	6.8 ± 0.5	6.8 ± 0.6	6.3 ± 0.3	6.4 ± 0.2	6.4 ± 0.2
SG	1.03 ± 0.02	1.03 ± 0.01	1.04 ± 0.02	1.04 ± 0.01	1.04 ± 0.02	1.03 ± 0.02
UGLU (mg/dL)	NEG	NEG	NEG	NEG	NEG	NEG
UBILI	NEG	NEG	NEG ^c	NEG	NEG	NEG
KET (mg/dL)	3.2 ± 6.6	0.2 ± 0.0	6.6 ± 7.7	NEG	NEG ^c	NEG ^c
URO (mg/dL)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
UPRO (mg/dL)	18.4 ± 15.9	30.0 ± 0.0	38.2 ± 36.8	12.4 ± 16.1	6.4 ± 13.2	6.2 ± 13.3
NIT	NEG	NEG ^c	NEG	NEG	NEG ^c	NEG ^c
LEU	NEG ^c	NEG	NEG ^c	NEG	NEG	NEG
BLD	NEG	NEG	NEG	NEG	NEG	NEG

VOL = volume, SG = specific gravity, UGLU = urinary glucose, UBILI = urinary bilirubin, KET = ketones, URO = urobilinogen, UPRO = urinary protein, NIT = nitrite, LEU = leucocytes, BLD = occult blood, NEG = negative, a = $p < 0.05$, b = $p < 0.01$, c = 1 animal positive.

2.5. Statistical analysis

Where appropriate, numeric data were evaluated statistically. For comparative statistics, data determined to be parametric were evaluated by univariate analysis of variance (ANOVA). If ANOVA verified significance at $p \leq 0.05$, pairwise

comparisons of treatment group(s) with the control group were made using a parametric test to identify statistical differences ($p \leq 0.05$). SYSTAT software (version 12) (Systat Software, Inc., San Jose, CA, USA) and Provantis software (version 8) (Instem LSS Ltd., Staffordshire, UK) were used for statistical analyses.

Table 5Hematological parameters after 90-day oral administration of NEM® (0, 400, and 2000 mg/kg bw/day) in rats. Data are shown as means ± standard deviations (*n* = 5).

	Male			Female		
	Dose (mg/kg body weight/d)			Dose (mg/kg body weight/d)		
	0	400	2000	0	400	2000
RBC (M/ μ L)	8.6 ± 0.7	9.1 ± 0.5	8.8 ± 0.2	8.1 ± 0.2	8.1 ± 0.6	8.3 ± 0.3
ABSRET (X10 ⁹ /L)	225 ± 45	206 ± 36	202 ± 39	148 ± 41	178 ± 56	194 ± 40
HGB (g/dL)	14.7 ± 1.0	15.6 ± 0.6	15.1 ± 0.6	14.8 ± 0.4	14.6 ± 0.7	15.0 ± 0.4
HCT (%)	45.4 ± 3.9	48.6 ± 2.1	46.5 ± 2.3	44.8 ± 0.8	44.4 ± 3.0	46.1 ± 1.2
MCH (pg)	17.0 ± 0.2	17.1 ± 0.6	17.3 ± 0.3	18.4 ± 0.2	17.9 ± 0.9	18.1 ± 0.4
MCHC (g/dL)	32.4 ± 0.8	32.1 ± 0.3	32.5 ± 1.1	33.1 ± 0.7	32.8 ± 0.8	32.6 ± 0.1
MCV (fL)	52.6 ± 0.7	53.3 ± 2.0	53.0 ± 1.6	55.4 ± 1.1	54.6 ± 2.1	55.6 ± 1.2
WBC (K/uL)	12.9 ± 3.7	15.3 ± 5.3	15.1 ± 5.0	4.0 ± 0.6	3.4 ± 0.6	4.1 ± 1.1
LYMPH (K/uL)	9.4 ± 3.3	12.1 ± 3.8	11.8 ± 4.6	3.3 ± 0.7	2.8 ± 0.5	3.4 ± 1.1
MONO (K/uL)	0.38 ± 0.14	0.48 ± 0.31	0.40 ± 0.16	0.11 ± 0.04	0.09 ± 0.02	0.09 ± 0.03
BAS (K/uL)	0.03 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	0.004 ± 0.006	0.0 ± 0.0	0.006 ± 0.006
EOS (K/uL)	0.16 ± 0.06	0.16 ± 0.06	0.17 ± 0.04	0.06 ± 0.03	0.05 ± 0.02	0.06 ± 0.02
NEUT (K/uL)	2.8 ± 1.4	2.4 ± 1.4	2.6 ± 1.4	0.5 ± 0.3	0.5 ± 0.1	0.6 ± 0.2

RBC = red blood cell count, ABSRET = absolute reticulocytes, HGB = hemoglobin, HCT = hematocrit, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, WBC = white blood cell count, LYMPH = lymphocytes, MONO = monocytes, BAS = basophils, EOS = eosinophils, NEUT = neutrophils, a = *p* < 0.05, b = *p* < 0.01.

Table 6Coagulation-related parameters after 90-day oral administration of NEM® (0, 400, and 2000 mg/kg bw/day) in rats. Data are shown as means ± standard deviations (*n* = 5).

	Male			Female		
	Dose (mg/kg body weight/d)			Dose (mg/kg body weight/d)		
	0	400	2000	0	400	2000
PLAT (K/uL)	1189 ± 247	962 ± 200	887 ± 127	1061 ± 161	957 ± 228	959 ± 281
MPV (fL)	8.2 ± 0.3	8.5 ± 0.5	8.2 ± 0.4	8.2 ± 0.2	8.4 ± 0.1	8.1 ± 0.4
PT (s)	20.9 ± 1.1	19.1 ± 0.6 ^a	20.3 ± 1.1	19.0 ± 1.8	18.4 ± 1.0	18.8 ± 0.9
APTT (s)	15.6 ± 1.3	14.9 ± 1.9	16.4 ± 1.5	15.8 ± 1.2	13.1 ± 1.6 ^b	13.9 ± 0.6 ^a

PLAT = platelets, MPV = mean platelet volume, PT = prothrombin time, APTT = activated partial thromboplastin time, a = *p* < 0.05, b = *p* < 0.01.

Table 7Clinical chemistry parameters after 90-day oral administration of NEM® (0, 400, and 2000 mg/kg bw/day) in rats. Data are shown as means ± standard deviations (*n* = 5).

	Male			Female		
	Dose (mg/kg body weight/d)			Dose (mg/kg body weight/d)		
	0	400	2000	0	400	2000
NA (mmol/L)	149 ± 2	150 ± 2	151 ± 2	148 ± 1	149 ± 1	150 ± 0
K (mmol/L)	6.2 ± 0.4	5.8 ± 0.4	6.3 ± 0.6	5.7 ± 0.6	6.4 ± 0.7	6.1 ± 0.8
CA (mg/dL)	11.7 ± 0.6	11.5 ± 0.7	12.1 ± 0.4	12.5 ± 0.7	13.0 ± 0.4	12.7 ± 0.6
CL (mmol/L)	100 ± 4	102 ± 3	103 ± 2	103 ± 2	102 ± 3	106 ± 4
GLU (mg/dL)	164 ± 48	192 ± 49	198 ± 38	168 ± 42	158 ± 21	165 ± 52
CREA (mg/dL)	0.52 ± 0.04	0.54 ± 0.05	0.50 ± 0.07	0.66 ± 0.05	0.64 ± 0.05	0.70 ± 0.19
TBILI (mg/dL)	0.10 ± 0.00	0.04 ± 0.05	0.08 ± 0.04	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
BUN (mg/dL)	16.0 ± 2.3	14.6 ± 3.5	13.0 ± 0.7	17.8 ± 3.3	14.2 ± 2.2	21.2 ± 9.0
TPRO (g/dL)	6.6 ± 0.5	6.6 ± 0.6	6.7 ± 0.2	7.8 ± 0.7	7.8 ± 0.3	7.5 ± 0.4
ALB (g/dL)	4.1 ± 0.2	4.2 ± 0.4	4.3 ± 0.2	5.0 ± 0.6	5.2 ± 0.2	4.8 ± 0.3
GLOB (g/dL)	2.5 ± 0.3	2.4 ± 0.2	2.4 ± 0.2	2.7 ± 0.2	2.7 ± 0.1	2.7 ± 0.2
A/G (Ratio)	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.2	1.8 ± 0.3	1.9 ± 0.1	1.8 ± 0.1
CHOL (mg/dL)	71 ± 10	63 ± 18	66 ± 21	96 ± 17	97 ± 35	81 ± 15
TRIG (mg/dL)	90 ± 37	79 ± 37	76 ± 24	56 ± 10	62 ± 24	55 ± 8
ALP (U/L)	92 ± 22	92 ± 16	105 ± 22	56 ± 17	56 ± 28	58 ± 21
LDH (U/L)	100 ± 26	128 ± 57	123 ± 43	87 ± 21	109 ± 60	92 ± 30
AST (U/L)	75 ± 11	89 ± 28	75 ± 9	97 ± 38	96 ± 55	85 ± 18
ALT (U/L)	35.8 ± 3.7	42.4 ± 9.1	35.6 ± 2.9	48 ± 23	45 ± 31	41 ± 12
GGT (U/L)	0.0 ± 0.0	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.6 ± 0.5
CK (U/L)	252 ± 224	276 ± 155	261 ± 144	135 ± 72	355 ± 385	215 ± 112
PHOS (mg/dL)	8.9 ± 1.1	9.1 ± 1.4	9.8 ± 0.5	8.3 ± 1.4	8.2 ± 1.8	9.0 ± 2.1

NA = sodium, K = potassium, CA = calcium, CL = chloride, GLU = glucose, CREA = creatinine, TBILI = total bilirubin, BUN = urea nitrogen, TPRO = total protein, ALB = albumin, GLOB = globulin, A/G = albumin/globulin ratio, CHOL = cholesterol, TRIG = triglycerides, ALP = alkaline phosphatase, lactate LDH = dehydrogenase, AST = aspartate aminotransferase, ALT = alanine aminotransferase, GGT = gamma-glutamyltransferase, CK = creatine phosphokinase, PHOS = inorganic phosphorus, a = *p* < 0.05, b = *p* < 0.01.

3. Results

3.1. Test article compositional analysis

Because of the complexity of many natural products, it is difficult to narrowly define their composition. For this reason, a number of nutritive and/or bioactive molecules are used collectively to define the composition of NEM®. Additional physical characteristics (i.e. particle size, bulk density, etc.) as well as microbiological contaminants are also utilized to characterize NEM. A typical compositional analysis would be: Total Protein – 40–60%, Collagen – 10–20%, Hyaluronic Acid – 0.5–2%, Chondroitin Sulfate – 0.2–1%, Particle Size – 2–6% retained on #60 mesh, Loose Density – 0.6–0.9 g/cc, Moisture – 3–8%, Total Plate Count – <5000 cfu/g, Yeasts and Molds – <100 cfu/g, *E. coli* – Negative/10 g, *Salmonella* – Negative/10 g, and *Staphylococcus aureus* – Negative/10 g.

3.2. Cytotoxicity evaluation

There was no inhibition in cell viability observed in test article dosed samples at any of the time points evaluated. Percent viability results from the comparison of NEM® to Triton X-100 are reported in Table 1. These data were then used to determine (by interpolation, if necessary) the time at which 50% viability (ET-50) is or would be reached. NEM® has an ET-50 >24 h, whereas Triton X-100 has an ET-50 of 2.3 h.

3.3. Mutagenicity evaluation

There were no significant revertant mutation rates observed exceeding the background average (spontaneous reversions) either with or without S9 metabolic activation in any of the bacterial strains assayed (see Table 2). Additionally, there was no dose-related increase in reversion rates over the range tested (50–5000 µg/plate).

The results of a repeated assay with 2.5× more S9 activator confirmed the negative results of the initial assay (data not shown).

3.4. Animal models of toxicology

3.4.1. Acute oral toxicity in rats

No deaths occurred during the 14-day post-treatment evaluation period. There were no treatment-related clinical signs of toxicity observed during the evaluation period nor was any weight loss observed in any animals. Finally, no treatment-related gross pathological changes were observed in any organs of the test animals during necropsy. The results of this evaluation show that the single oral dose resulted in an LD₅₀ of greater than 2000 mg/kg bw and indicates a low order of acute toxicity.

3.4.2. 90-day oral toxicity in rats

No deaths occurred during the 90-day evaluation period. There were no treatment-related clinical signs of toxicity observed during the evaluation period. There were no statistically significant differences in average daily food consumption (Fig. 1) or body weight gain (Fig. 2) between control and treatment groups during the evaluation period.

Organ weights, organ/body weight ratios, and organ/brain weight ratios were within normal ranges for all test animals (Table 3). There were a small number of minor organ weight variations that were not dose-dependent and were not considered treatment-related effects. There was a statistically significant difference in mean absolute ovary weight ($p < 0.05$) and ovary/brain weight ratio ($p < 0.05$) between the control and 2000 mg/kg bw/day treatment group. Due to the possibility of a treatment-related effect, ovaries from the 2000 mg/kg bw/day treatment group were included in the histopathological evaluation. There were no correlative abnormal histomorphologic findings upon examination and the mean weights were within historic controls for the conducting lab. Therefore, the increased ovary weight was considered a result

Table 8

Summary of histopathological findings after 90-day oral administration of NEM®. Listed organs are only those with findings, however all organs and tissues described in the study protocol were examined. Data are shown as number of animals with findings/number of animals examined.

	Male			Female		
	Dose (mg/kg body weight/d)			Dose (mg/kg body weight/d)		
	0	400	2000	0	400	2000
<i>Adrenal glands</i>						
Minimal diffuse bilateral congestion	0/5	–	0/5	1/5	–	1/5
Cervical lymph node						
Dark red discoloration	1/5	0/5	1/5	0/5	0/5	0/5
<i>Heart</i>						
Minimal mononuclear cell infiltration	1/5	–	3/5	0/5	–	1/5
Mild mononuclear cell infiltration	1/5	–	1/5	0/5	–	0/5
<i>Kidneys</i>						
Minimal chronic progressive nephropathy	2/5	–	2/5	2/5	–	1/5
<i>Liver</i>						
Minimal mononuclear cell infiltration	4/5	–	4/5	3/5	–	5/5
<i>Lungs</i>						
Minimal acute hemorrhage	3/5	–	2/5	1/5	–	1/5
Marked acute multifocal hemorrhage	0/5	–	1/5	0/5	–	0/5
Pleural lymphoplasmacytic inflammation	0/5	–	0/5	1/5	–	0/5
<i>Ovaries</i>						
Decreased corpora lutea	–	–	–	1/5	1/5	0/5
Pancreas						
Mild subacute interstitial inflammation	1/5	–	0/5	0/5	–	0/5
<i>Thymus</i>						
Dark red pinpoint discoloration	3/5	1/5	3/5	0/5	0/5	0/5

– = No data.

of individual animal variation and not a treatment-related effect. Urinalysis results were unremarkable (Table 4). Hematological evaluation was unremarkable (Table 5). Coagulation analysis presented minor differences that were not dose-dependent and were therefore not considered treatment-related effects (Table 6). Clinical chemistry evaluation was unremarkable (Table 7). Upon gross pathological evaluation at the end of the study period, there were a number of minor findings (Table 8) that presented in both the control and treatment animals, and so were not deemed a treatment-related effect. Based on the results of this study the NOEL (No Observable Effect Level) is 2000 mg/kg bw/day (the highest dose level administered).

4. Discussion

NEM[®] has been shown to naturally contain a number of components such as glucosamine, chondroitin sulfate, dermatan sulfate, hyaluronic acid, collagen, etc. that are found in joints and connective tissues and are thought to be beneficial when consumed. Recently, NEM[®] has been shown to be clinically effective at 500 mg per day in reducing joint pain and stiffness (Ruff et al., 2009b) and increasing flexibility (Ruff et al., 2009a) in a number of human trials. As evidence increases to support NEM[®] as a natural therapeutic for osteoarthritis and other diseases of the joints and connective tissues, it is important to demonstrate through peer-reviewed publication that adequate safety studies have previously been conducted to support the use of this product in dietary supplements or food products that are expected to be consumed chronically by humans. Although a food-based product such as eggshell membrane would be expected to be inherently safe, the source of bioactivity for NEM[®] remains to be determined. And so a safety evaluation was initiated to further support its anticipated consumption.

None of the known constituents of eggshell membrane have been previously found in the literature to be substantially cytotoxic independently in normal cellular assays (Anderson et al., 2005; Necas et al., 2008), however, it was important to demonstrate a lack of cytotoxicity collectively, and also that an as yet unknown constituent of eggshell membrane would not prove to be cytotoxic. NEM[®] did not exhibit signs of significant cytotoxicity in a human-derived epidermal keratinocyte *in vitro* assay, reported herein. This is supported by findings from a recent *in vitro* study (unpublished results) involving human peripheral blood mononuclear cells (PBMCs) in which there was also no indication of cytotoxicity from NEM[®] exposure.

As eggshell membrane plays a substantial role in embryonic development in a fowl, there would be no reason to expect eggshell membrane or its derivatives to possess mutagenic activity. This is indeed what was found. NEM[®] did not exhibit signs of significant mutagenicity (Ames Reverse Mutation Study) either in the presence or absence of a metabolic activator. NEM[®] was evaluated over a fairly broad concentration range (50–5000 µg/plate) with no indication of a dose-related mutagenic effect, including a repeated assay with 2.5× increased metabolic activator.

People likely ingest small amounts of eggshell membrane when consuming hard-boiled and soft-boiled eggs, as the membrane can sometimes be difficult to avoid completely during consumption. There have been no reports in the literature of toxicity from this low-level, chronic (albeit intermittent) ingestion of eggshell membrane. Again, this is not unexpected from food-based materials, and this would be expected to hold true for more substantial quantities of eggshell membranes or its derivatives. Additionally, in light of the lack of cytotoxicity and mutagenicity *in vitro*, NEM[®] would be expected to have similar properties *in vivo*. This was indeed found to be the case. NEM[®] exhibited no significant clinical or

pathological signs of toxicity in either an acute or subchronic repeated oral toxicological evaluation. Acute toxicity was initially evaluated at up to 2000 mg/kg body weight without the observation of any adverse effects. Based upon this finding, the 90-day repeated dose toxicological evaluation was carried out at 400 mg/kg bw/day and 2000 mg/kg bw/day. Using an allometric inter-species method of conversion that normalizes doses based upon body surface area, the 90-day repeated dose levels evaluated in rats correspond to approximately 10× and 50× the human equivalent dose (HED) of 500 mg per day, respectively (U.S. Food and Drug Administration, 2005). Even at the highest dose tested, there were no signs of toxicity that were related to administration of the test article in any of the parameters evaluated.

4.1. Conclusion

Natural Eggshell Membrane (NEM[®]) is a novel dietary ingredient that contains naturally occurring glycosaminoglycans and proteins essential for maintaining healthy joint and connective tissues and has shown considerable promise in recent human clinical trials at a dose of 500 mg per day. It is therefore important to demonstrate to the public that an adequate safety evaluation has been previously conducted to support the use of NEM[®] in dietary supplements or food products that are expected to be consumed on a continuing basis. NEM[®] was evaluated for cytotoxicity, genotoxicity, and oral toxicity (single acute dose and 90-day repeated-dose) at doses up to fifty times (50X) the clinically tested human equivalent dose. The results of these studies indicate that NEM[®] is safe as a supplement for human consumption at levels up to 500 mg/day.

Conflict of Interest

J.K. Ruff is an employee of EMS Technologies. All other authors declare no conflicts of interest.

References

- Adams, R.G., Franklin, M.R., 2006. Vacuum treatment of an input stream without ruining delicate output fractions, United States Patent 7017, 277 B1.
- Anderson, J.W., Nicolosi, R.J., Borzelleca, J.F., 2005. Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. *Food Chem. Toxicol.* 43 (2), 187–201.
- Association of American Feed Control Officials, 2009. Section 9.0 – Animal products, in: AAFCO Official Publication. Association of American Feed Control Officials Incorporated, pp. 341–348 (Chapter 5).
- Baker, J.R., Balch, D.A., 1962. A study of the organic material of hen's-egg shell. *Biochem. J.* 82, 352–361.
- Freedman, R.L., Human Food Uses: A Cross-Cultural, Comprehensive Annotated Bibliography. Greenwood Press, Westport Connecticut, 1981, pp. 46, 248, 429.
- Hirasawa, T., Omi, N., Ezawa, I., 2001. Effect of 1α-hydroxyvitamin D₃ and egg-shell calcium on bone metabolism in ovariectomized osteoporotic model rats. *J. Bone Miner. Metab.* 19, 84–88.
- Long, A.L., 1913. Egg delicacies of the orient. *Pure Products* 9, 494–496.
- Long, F.D., Adams, R.G., DeVore, D.P., 2005. Preparation of hyaluronic acid from eggshell membrane. USA Patent 6946, 551.
- Necas, J., Bartosikova, L., Brauner, P., Kolar, J., 2008. Hyaluronic acid (hyaluronan): a review. *Vet. Med.* 53 (8), 397–411.
- Picard, J., Paul-Gardais, A., Vedel, M., 1973. Sulfated glycoproteins from egg shell membranes and hen oviduct. Isolation and characterization of sulfated glycopeptides. *Biochim. Biophys. Acta* 320, 427–441.
- Ruff, K.J., DeVore, D.P., Leu, M.D., Robinson, M.A., 2009a. Eggshell membrane: a possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin. Interv. Aging* 4, 235–240.
- Ruff, K.J., Winkler, A., Jackson, R.W., DeVore, D.P., Ritz, B.W., 2009b. Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clin. Rheumatol.* 28, 907–914.
- Schaafsma, A., Beelen, G.M., 1999. Eggshell powder, a comparable or better source of calcium than purified calcium carbonate: piglet studies. *J. Sci. Food Agric.* 79, 1596–1600.

- Schaafsma, A., Pakan, I., 1999. Short-term effects of a chicken egg shell powder enriched dairy-based product on bone mineral density in persons with osteoporosis or osteopenia. *Bratisl. Lek. Listy* 100, 651–656.
- Stancikova, M., Masaryk, P., Letkovska, A., Rovensky, J., Wendlova, J., Orvisky, E., 1996. Effect of biomin H on the biochemical markers of osteoporosis in postmenopausal women. *Osteoporosis Int.* 6, 267.
- Svik, K., Stancikova, M., Masaryk, P., Rovensky, J., Veselkova, Z., 1996. Effect of biomin H and vitamin D₂ on the ovariectomized rats. *Osteoporosis Int.* 6, 266.
- Tsai, W.T., Yang, J.M., Lai, C.W., Cheng, Y.H., Lin, C.C., Yeh, C.W., 2006. Characterization and adsorption properties of eggshells and eggshell membrane. *Bioresour. Technol.* 97, 488–493.
- United Nations Food and Agriculture Organization, 2004. *FAO Statistical Yearbook – United States of America*.
- U.S. Food and Drug Administration, 2005. Guidance for industry: estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. Downloaded from: <<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>>.
- Wong, M., Hendrix, M.J.C., von der Mark, K., et al., 1984. Collagen in the egg shell membranes of the hen. *Dev. Biol.* 104, 28–36.

Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study

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Abstract Natural Eggshell Membrane (NEM®) is a new novel dietary supplement that contains naturally occurring glycosaminoglycans and proteins essential for maintaining healthy articular cartilage and the surrounding synovium. The randomized, multicenter, double-blind, placebo-controlled Osteoarthritis Pain Treatment Incorporating NEM® clinical study was conducted to evaluate the efficacy and safety of NEM® as a treatment for pain and stiffness associated with osteoarthritis of the knee. Sixty-seven patients were randomly

assigned to receive either oral NEM® 500 mg ($n=34$) or placebo ($n=33$) daily for 8 weeks. The primary endpoint was the change in overall Western Ontario and McMasters Universities (WOMAC) Osteoarthritis Index as well as pain, stiffness, and function WOMAC subscales measured at 10, 30, and 60 days. The clinical assessment was performed on the intent-to-treat population. Supplementation with NEM® produced an absolute rate of response that was statistically significant (up to 26.6%) versus placebo at all time points for both pain and stiffness, but was not significantly improved for function and overall WOMAC scores, although trending toward improvement. Rapid responses were seen for mean pain subscores (15.9% reduction, $P=0.036$) and mean stiffness subscores (12.8% reduction, $P=0.024$) occurring after only 10 days of supplementation. There were no serious adverse events reported during the study and the treatment was reported to be well tolerated by study participants. Natural Eggshell Membrane (NEM®) is an effective and safe option for the treatment of pain and stiffness associated with knee osteoarthritis. Supplementation with NEM®, 500 mg taken once daily, significantly reduced both joint pain and stiffness compared to placebo at 10, 30, and 60 days. The Clinical Trial Registration number for this study is NCT00750477.

Keywords Alternative · Complimentary · Eggshell membrane · Knee · OPTION · Osteoarthritis · Pain · Stiffness · WOMAC

Introduction

Osteoarthritis (OA) is the most prevalent form of arthritis and is estimated to affect nearly 27 million adults in the U.S., with one-third of those 65 and older having been diagnosed

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with OA [1]. As the population ages, this estimate is expected to grow rapidly. Traditional treatments for OA usually involve the use of analgesics (i.e., acetaminophen, tramadol), non-steroidal anti-inflammatory drugs (NSAIDs) (i.e., ibuprofen, diclofenac), or cyclooxygenase-2-specific (COX-2) NSAIDs (i.e., celecoxib) alone or in combination. Steroid and hyaluronic acid injections have also been used with some success. Many of these treatments have shown limited effectiveness in randomized controlled clinical trials (RCTs) [2–5]. To avoid the cardiac risks [6–9] and gastrointestinal issues [10, 11] associated with traditional OA treatments (particularly with long-term use), many patients have turned to complementary and alternative medicines (CAMs) such as dietary supplements.

Glucosamine and chondroitin, alone and in combination, are widely marketed as dietary supplements to treat joint pain due to OA. There have been two major human clinical trials that have investigated the role of these two dietary supplements in the treatment of OA symptoms. The Glucosamine/chondroitin Arthritis Intervention Trial (GAIT), a 1,583-patient, 6-month trial sponsored by the National Institutes of Health (NIH), failed to show significant improvement in the Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index in the overall patient population for glucosamine, chondroitin, or their combination [12]. The Glucosamine Unum In Die (once-a-day) Efficacy (GUIDE) trial, a 318-patient, 6-month European trial sponsored by industry, showed a small, 5–6% improvement in total WOMAC Index score over placebo for glucosamine sulfate [13]. Because of their limited effectiveness, the search for additional CAMs to treat OA continues.

In the U.S. alone, an estimated 600,000 tons of eggshells are produced annually as a by-product of the poultry industry [14]. Disposal of these eggshells creates an environmental and financial burden and, therefore, alternative uses for these materials would be of obvious benefit. Eggshell membranes are primarily composed of fibrous proteins such as collagen type I [15]. However, eggshell membranes have also been shown to contain glycosaminoglycans (GAGs), such as dermatan sulfate and chondroitin sulfate [16], hexosamines, such as glucosamine, as well as hexoses and fucose [17]. More recently, significant amounts of hyaluronic acid have been detected in eggshell membrane [18]. Other components identified in eggshell membrane include sialic acid [19], desmosine and isodesmosine [20], ovotransferrin [21], lysyl oxidase [22], and lysozyme [23].

The discovery of eggshell membrane as a natural source of combined glucosamine, chondroitin, and hyaluronic acid has prompted the evaluation of this material as a potential treatment for OA. ESM Technologies, LLC (Carthage, MO, USA) has developed methods to efficiently and effectively separate eggshell membrane from eggshells to create a

shell-free eggshell membrane. The isolated membrane is then partially hydrolyzed using a proprietary process and dry blended to produce 100% pure Natural Eggshell Membrane (NEM®).

In preliminary open-label human clinical trials totaling 37 subjects with joint and connective tissue disorders, oral supplementation with 500 mg per day of NEM® resulted in an observed decrease in pain in 7–30 days (unpublished report). Therefore, an 8-week randomized, multicenter, double-blind, placebo-controlled supplementation trial was conducted to evaluate the efficacy and safety of NEM® for the relief of the pain and stiffness associated with moderate OA of the knee—the Osteoarthritis Pain Treatment Incorporating NEM® (OPTION) trial. The results are presented herein.

Patients and methods

Study design

The OPTION study was conducted according to a randomized, multicenter, double-blind, placebo-controlled design and was conducted in three rheumatology clinics in Missouri (USA) in accordance with the U.S. Food & Drug Administration's principles of Good Clinical Practice (Title 21, Code of Federal Regulations, Parts 50 & 56 and ICH E6) and the Declaration of Helsinki. The study protocol was approved by an independent regional institutional review board and patients provided their written informed consent to participate. Subjects were required to suspend all current pain relief medications in order to participate in the study. Eligible subjects were then centrally randomized among all sites to receive either NEM® or placebo in the order in which they were enrolled in the study using a permuted-block randomization table consisting of four subjects per block. Patients, clinical investigators, and the study coordinator were all blinded to the treatment through the completion of the study. Treatment consisted once daily orally of either NEM® (Membrell, LLC, USA) or placebo (excipients) provided in 500 mg vegetarian capsules that were stored in closed containers at ambient temperature. Clinic visits were scheduled for subjects at 10, 30, and 60 days following the onset of treatment. Treatment compliance was checked at clinic visits by patient interview and by counting the number of unused doses of the study medications. Acetaminophen was allowed for pain relief rescue, if necessary. Subjects recorded the time and amount of acetaminophen taken in patient diaries.

Patients

All subjects 18 years of age or older with known symptomatic osteoarthritis of the knee were considered for

enrollment in the study. In order to be eligible, patients must have been diagnosed with functional grades I–III of osteoarthritis according to the modified criteria of the American College of Rheumatology [24]. Subjects must also have had persistent knee pain associated with osteoarthritis with a baseline score of at least 30 mm on the Patient's Assessment of Arthritis Pain—Visual Analog Scale. Subjects were required to suspend all current pain relief medications. Subjects that were currently taking analgesic medications were eligible to participate in the study following a 14-day washout period for NSAIDs, a 7-day washout for narcotics, and a 90-day washout for injected steroids. Subjects currently taking glucosamine, chondroitin sulfate, or MSM were only eligible after a 3-month washout period. Patients were excluded if they were currently receiving remission-inducing drugs such as methotrexate or immunosuppressive medications or had received them within the past 3 months. They were also excluded if they had a confounding inflammatory disease or condition (rheumatoid arthritis, gout, pseudo gout, Paget's disease, chronic pain syndrome, etc.) that would interfere with assessment of pain associated with the index knee. Other exclusionary criteria were: body weight 113.5 kg (250 lbs) or greater, a known allergy to eggs or egg products, or pregnant or breastfeeding women. Subjects previously enrolled in a study to evaluate pain relief within the past 6 months or currently involved in any other research study involving an investigational product (drug, device, or biologic) or a new application of an approved product, within 30 days of screening, were also excluded from participating in the trial.

Treatment response

The primary endpoint of the study was measurement of the effectiveness of NEM® in relieving pain, stiffness, and discomfort associated with moderate OA of the knee and to compare its effectiveness to placebo. The primary treatment response endpoints were the 10-, 30-, and 60-day clinic assessments utilizing the Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index—Visual Analog Scale (100 mm) version (VA 3.1) [25]. This version of the WOMAC questionnaire consists of five questions addressing the severity of joint pain, two questions addressing joint stiffness, and 17 questions addressing limitations in performing physical activities (function). Endpoints were compared to pretreatment assessments and to placebo controls. In 2004, the Outcome Measures in Rheumatology Clinical Trials (OMERACT) and the Osteoarthritis Research Society International (OARSI) published criteria for a response to treatment for osteoarthritis [26]. A treatment response was classified as an improvement in pain or function of at least 50% and a decrease of at least 20 mm on

the visual-analog scale for pain or function (WOMAC subscales). Alternatively, the occurrence of two of the following criteria also was acceptable as a treatment response: a decrease in pain of at least 20% and at least 10 mm on the visual-analog scale; an improvement in function of at least 20% and a decrease of at least 10 mm on the visual-analog scale; and an increase in the patient's global assessment score by at least 20% and at least 10 mm on the visual-analog scale. Patient's global assessment scores were not collected using a visual-analog scale; however, we only review the OMERACT–OARSI response rate with respect to the primary criterion. Patient's and Physician's Global Assessments of Arthritis were also collected utilizing a 0–5-point Likert scale.

Adverse events

Secondary objectives of the study were to evaluate tolerability and any adverse reactions associated with supplementation with NEM®. The subjects' self-assessment diaries were reviewed and any discomfort or other adverse events were recorded and reported in accordance with applicable FDA regulations. Adverse events and serious adverse events were assessed by the clinical investigator at each study visit and followed until resolution, as necessary. Serious adverse events were required to be reported to the clinical monitor immediately using MedWatch OMB No. 0910-0291.

Statistical analysis

An absolute increase in the mean response rate of 35% (treatment rate versus placebo rate) was considered a clinically meaningful treatment effect. It was estimated that a sample size of 75 patients would need to be enrolled to provide the study with a statistical power of 85% to detect a clinically meaningful difference between the treatment group and the placebo group, assuming a rate of response of 40% in the treatment group, a rate of response of 5% in the placebo group, and a withdrawal rate of 20%. Pairwise comparisons of the treatment group with the placebo group were made with a two-sided independent group *t* test at baseline to validate randomization. Within-group comparisons, using a two-sided independent group *t* test, were also made between testing sites to rule out any site bias. In both cases, statistical significance was accepted at an α value of <0.05 . Post-baseline statistical analyses were done utilizing repeated measures univariate analysis of variance (RM-ANOVA) on pooled population data. Statistical significance was accepted at an α value of <0.05 for between-group interactions. Analysis of the primary outcome measure (the difference between groups and the change from baseline in overall WOMAC composite score as well as pain, stiffness,

and function subscores) was conducted in the intent-to-treat (ITT) population (i.e., including all randomized patients with at least one efficacy assessment after randomization). The last observation carried forward (LOCF) approach was used for patients who made at least one follow-up visit but who did not complete the study (lost to follow-up). A per-protocol completer analysis was also performed. SYSTAT software (version 12) was used for all statistical analyses [27].

Results

Patient recruitment began in December 2004 at three clinical sites in Missouri and the final follow-up was conducted in January 2006. A total of 67 subjects were enrolled in the trial and underwent randomization (see Fig. 1). Of these subjects, 61.1% were from site 1, 29.9% from site 2, and 9.0% from site 3. In terms of OA functional grades, 20.9% were grade I, 28.4% were grade II, 20.9% were grade III, and 29.9% were unassigned. Seven subjects did not complete baseline evaluations, resulting in a total of 60 subjects in the intent-to-treat (ITT) population. Thirty-one subjects (51.6%) were randomized to the placebo group and 29 subjects (48.3%) were randomized to the NEM[®] treatment group. Thirty-one percent (31%) of the ITT subjects assigned to NEM[®] did not complete the 2-month study per the protocol, compared with 42% of the ITT subjects assigned to placebo. Of the 60 subjects in the ITT population, six subjects assigned to placebo and two

subjects assigned to NEM[®] either violated the protocol or did not begin treatment and, therefore, were not available for further analysis. Those patients lost to follow-up before the first evaluation time point in both the placebo (four patients) and treatment (three patients) groups had symptomatically mild OA (mean WOMAC 39.7 and 45.6, respectively). Those patients lost to follow-up (primarily withdrawals) in the remainder of the study in both the placebo (three patients) and the treatment (four patients) groups had symptomatically more severe OA (mean WOMAC 76.6 and 63.7, respectively) compared to those patients that completed the study (mean WOMAC at baseline of 52.6 and 45.3, respectively). Five patients in the placebo group and four patients in the treatment group officially withdrew from the study due to lack of efficacy. There were no obvious differences in the reason for withdrawal between the study groups. The overall drop-out rate of 43% (from enrollment) was considerably higher than the estimated rate of 20%, although >70% of the subjects that began treatment ($n=54$) completed the study (excluding non-compliance). This is possibly related to the small sample population or could also be due to the relatively stringent pain management requirements of the study protocol. Compliance with the study treatment regimen was good in both treatment groups. In those subjects that completed the study, the rate of compliance was >97% (as judged by capsule count at clinic visits).

Patient data was initially evaluated to ensure randomization within each site. Additionally, patient data was

Fig. 1 Enrollment, randomization, and completion flow diagram

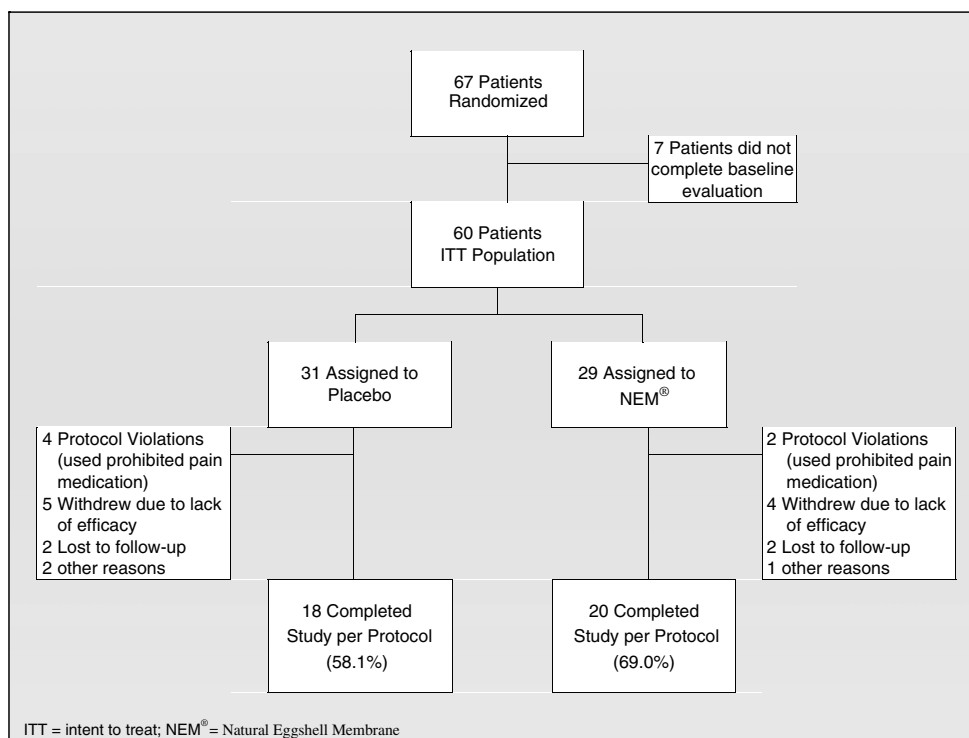


Table 1 Mean WOMAC scores by category in NEM-supplemented and control groups at baseline and 10, 30, and 60 days post-treatment

	Days post-treatment	Treatment		<i>P</i> value
		Placebo	NEM	
Pain	Baseline (<i>n</i> =25, 25)	50.6±19.4	44.0±16.8	0.204
	10 (<i>n</i> =21, 24)	52.7±24.1	39.0±19.4	0.036*
	30 (<i>n</i> =21, 24)	53.7±21.0	42.3±26.2	0.040*
	60 (<i>n</i> =21, 24)	50.7±22.2	37.5±25.2	0.038*
Stiffness	Baseline (<i>n</i> =25, 25)	59.3±24.0	50.5±20.3	0.167
	10 (<i>n</i> =21, 24)	57.0±25.6	42.5±25.0	0.024*
	30 (<i>n</i> =21, 24)	60.6±23.0	43.5±23.5	0.009*
	60 (<i>n</i> =21, 24)	56.5±24.3	35.0±25.8	0.005*
Function	Baseline (<i>n</i> =25, 25)	55.2±21.3	48.1±19.5	0.227
	10 (<i>n</i> =21, 24)	57.3±24.6	43.3±23.0	0.084
	30 (<i>n</i> =21, 24)	55.6±21.8	45.1±25.5	0.079
	60 (<i>n</i> =21, 24)	53.1±24.9	40.5±27.1	0.076
Overall	Baseline (<i>n</i> =25, 25)	54.6±20.4	47.5±17.5	0.191
	10 (<i>n</i> =21, 24)	56.2±24.1	42.3±21.6	0.059
	30 (<i>n</i> =21, 24)	55.5±21.4	44.4±25.1	0.055
	60 (<i>n</i> =21, 24)	52.9±23.9	39.4±26.1	0.052

Values represent means±standard deviations. *P* values were determined by repeated measures univariate analysis of variance (RM-ANOVA), and represent treatment versus placebo

**P*<0.05

evaluated between sites to exclude site bias. Due to the lack of a characteristic placebo effect in the overall study population, blinding was further scrutinized within the placebo group. When evaluating the placebo group by site and month enrolled, the placebo effect that was observable was evenly distributed over time and between sites. That is, there was a relevant placebo effect in six of the eight months of enrollment across all sites. As there were no observable systematic abnormalities in any of these evaluations, the data were pooled for all subsequent analyses. A clinical comparison of valid (excluding non-compliance) subjects was carried out to obtain mean baseline values (see Table 1). In all cases, the treatment group values were slightly lower than those of the control group, but were not statistically different. Analysis of the

primary outcome measure revealed that supplementation with NEM[®] produced an absolute rate of response that was significantly better (ranging from 10.3% to 26.6% improvement) than placebo at all time points for both pain and stiffness, but fell short of significance for function and overall WOMAC, despite improving by 8.8% to 15.5% (see Table 2). There were rapid responses seen for mean pain subscores (15.9% reduction, *P*=0.036) and mean stiffness subscores (12.8% reduction, *P*=0.024) occurring after only 10 days of supplementation. At 60 days, pain response was maintained (15.4%, *P*=0.038), while stiffness had improved further to 26.6% reduction (*P*=0.005). Mean function subscores showed a 15.5% (*P*=0.084) absolute improvement versus placebo at 10 days, which fell slightly to 13.5% (*P*=0.076) by day 60. Overall mean WOMAC

Table 2 Mean treatment effect (%) in WOMAC scores from baseline by category in NEM-supplemented and control groups at 10, 30, and 60 days post-treatment

	Days post-treatment	Placebo	NEM	Absolute treatment effect
Pain	10 (<i>n</i> =21, 24)	+4.2%	-11.7%	-15.9%
	30 (<i>n</i> =21, 24)	+6.0%	-4.3%	-10.3%
	60 (<i>n</i> =21, 24)	+0.1%	-15.3%	-15.4%
Stiffness	10 (<i>n</i> =21, 24)	-3.9%	-16.7%	-12.8%
	30 (<i>n</i> =21, 24)	+2.2%	-14.6%	-16.8%
	60 (<i>n</i> =21, 24)	-4.7%	-31.3%	-26.6%
Function	10 (<i>n</i> =21, 24)	+3.9%	-11.6%	-15.5%
	30 (<i>n</i> =21, 24)	+0.8%	-8.0%	-8.8%
	60 (<i>n</i> =21, 24)	-3.8%	-17.3%	-13.5%
Overall	10 (<i>n</i> =21, 24)	+2.9%	-12.3%	-15.2%
	30 (<i>n</i> =21, 24)	+1.7%	-7.9%	-9.6%
	60 (<i>n</i> =21, 24)	-3.1%	-18.2%	-15.1%

Values are mean treatment differences calculated from Table 1 and are based upon the LOCF approach. Negative values represent improvement or reduction in symptom and positive values represent the inverse

scores resulted in a 15.2% ($P=0.059$) absolute improvement versus placebo at 10 days, which was maintained at 60 days (15.1%, $P=0.052$). Thirty-two percent (32%) of patients in the treatment group demonstrated a primary OMERACT–OARSI response versus 12% of patients in the placebo group by the end of the follow-up period (60 days, $P=0.023$).

The study population was too small to stratify the patients according to covariates, such as baseline pain level or grade (I–III), to obtain statistically relevant data. There was little difference between response rates for patients with more severe OA (grades II/III) compared with patients with less severe OA (grade I). About one-third of the patients in both groups had at least a 40% reduction in pain at 60 days.

Overall, the use of rescue pain medication was low throughout the study, occurring once in every 6–8 days. There were no significant differences among the groups for rescue medication use. Approximately 17% of subjects took acetaminophen significantly more often than average (once every 3 days or less). After reviewing the patient diaries, it was noted that many of the instances of acetaminophen use were not for rescue purposes, but were actually for headaches, backaches, and other issues unrelated to the treatment knee. There were three adverse events reported during the study and none of them were judged by clinical investigators to be associated with treatment. There were no serious adverse events reported during the study. Of particular note is that there were no allergy-associated adverse events during the study, although those with known egg allergies were excluded from participating during screening. In general, the treatment was reported to be extremely well tolerated by study participants.

Discussion

Osteoarthritis is extremely prevalent and results in significant costs, both financial and quality of life, for those that suffer from the debilitating disease. The OPTION trial was designed to evaluate the efficacy and safety of Natural Eggshell Membrane as a treatment option for osteoarthritis. Our preliminary study indeed proved NEM® both effective

and safe for treating pain and stiffness associated with OA of the knee. NEM® has the added benefit of avoiding the concerning side effects associated with long-term use of other OA treatments such as NSAIDs.

Patients experienced a relatively rapid (10 days) response for all WOMAC scores with a mean response of approximately 15% (12.8% to 15.9%). By the end of the follow-up period (60 days), the mean response remained approximately 15% (13.5% to 15.4%) for all WOMAC scores except stiffness which was 26.6%. While this is superior to the response shown for glucosamine and chondroitin in previous clinical investigations [12, 13], it failed to reach the expected 35% response rate employed in the clinical design. Despite this shortcoming, the results were shown to be statistically significant. The safety profile for NEM® is also of significance as there are no known side effects, excluding the obvious egg allergy concern. This is of obvious importance in a condition that requires long-term treatment. Significant and sometimes serious side effects associated with other OA treatments frequently limits treatment options.

The measure of subjective symptoms (i.e., pain, stiffness, etc.) of arthritis and the wide variation in individual patient's perception of these symptoms results in complex relationships that can be difficult to elucidate from the reporting of mean treatment effects in clinical trials and may fail to adequately describe the potential benefits to the individual patient [28–31].

Number Needed to Treat (NNT) is a form of responder analysis and is a widely accepted and statistically valid measure of treatment effect [32]. NNTs of 5 or below are generally accepted as equating to an effective treatment for pain-related conditions [30]. In order to perform an NNT evaluation of the OPTION data, a treatment response rate table was prepared for the treatment and placebo groups at all time points for the pain (see Table 3) and stiffness (not shown) WOMAC subscales. It becomes evident that there are response rates that are quite likely to be clinically relevant (i.e., $\geq 30\%$ reduction from baseline). For example, approximately one-third (33%) of study subjects experienced greater than 30% reduction in pain at 10 days, with a similar number of subjects (32%) having experienced greater than 50% reduction in pain at 60 days. In both

Table 3 Percent of patients experiencing reduction in pain from Baseline at 10, 30, and 60 days post-treatment

% Reduction	10days		30days		60days	
	Placebo <i>n</i> =21	Treatment <i>n</i> =24	Placebo <i>n</i> =20	Treatment <i>n</i> =22	Placebo <i>n</i> =18	Treatment <i>n</i> =19
≥ 20	24%	54%	35%	32%	39%	67%
≥ 30	14%	33%	20%	23%	33%	42%
≥ 40	10%	17%	10%	23%	22%	42%
≥ 50	5%	8%	5%	23%	12%	32%

instances, this rate was more than two times ($\sim 2.5\times$) that of the placebo group. Approximately one-quarter (25%) of study subjects experienced greater than 50% reduction in stiffness at 10 days, with the number of patients increasing to more than one-half (53%) having experienced this level of improvement at 60 days. The 10-day result was more than two times ($\sim 2.5\times$) that of the placebo group and the 60-day result was nearly five times ($\sim 4.8\times$) that of placebo.

These various responder rates were then converted to NNT values which include 95% confidence intervals (95% CI) according to the method described by Wen et al. [33]. NNT values were determined for each level of improvement (as shown in Table 3) for both pain and stiffness. At 10, 30, and 60 days, NNTs for at least 50% reduction in pain were 28.0 (95% CI, 26.2 to 29.8), 5.6 (3.9 to 7.4), and 5.0 (3.1 to 6.9), respectively. In clinical practice, one out of every five patients should experience at least a 50% reduction in pain within 30–60 days. By comparison, we determined an NNT of 23.8 (95% CI, 15.2 to 32.4) from the GAIT data for a 50% reduction in WOMAC pain scores for the overall study population [12]. A similar 50% reduction in rheumatoid arthritis pain was reported as 4 in a review of three clinical trials for adalimumab, etanercept, and double-dose infliximab [34].

NNT values were also determined for 50% reduction in stiffness at each time point. We obtained NNTs of 6.5 (95% CI, 4.6 to 8.4), 7.9 (6.1 to 9.7), and 2.4 (0.5 to 4.3) at 10, 30, and 60 days, respectively. This demonstrates that there is a clinically relevant reduction in stiffness at all time points during the study. This is particularly true at 60 days where nearly one out of every two patients would experience a 50% reduction in stiffness.

With one-third of those 65 and older having been diagnosed with osteoarthritis [1], and that number expected to grow immensely as the overall US population ages, it is important for patients to have treatment options that are both effective and safe. The reporting of the results from the OPTION trial provides this needed treatment option.

The trial suffered from a number of issues. The limited initial enrollment (67 subjects), the relatively high drop-out rate (43%), and the smaller mean treatment effect than anticipated (15% versus 35%) could have compromised the statistical significance of the trial results. In addition to these inherent limitations, combined they also prevented *post hoc* analysis of subgroups of patients, say by severity of disease. Less stringent requirements for concomitant pain treatment or a more liberal rescue pain policy may have reduced the drop-out rate considerably. The inclusion of a comparative treatment agent may have provided additional information, but would have required a significantly larger study population. A larger follow-up study with some modifications may allow us to better determine which patients are most helped by NEM[®] supplementation.

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Disclosures KJR is currently employed by the sponsor of the study. AW and RWJ have no competing interests. DPD and BWR have served as paid consultants for the sponsor of the study.

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References

1. Lawrence RC, Felson DT, Helmick CG et al (2008) Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. *Arthritis Rheum* 58(1):26–35
2. Case JP, Baliunas AJ, Block JA (2003) Lack of efficacy of acetaminophen in treating symptomatic knee osteoarthritis. *Arch Intern Med* 163:169–178
3. Towheed TE, Maxwell L, Judd MG, Catton M, Hochberg MC, Wells G (2006) Acetaminophen for osteoarthritis. *Cochrane Database of Systematic Reviews*, No. 2
4. Geba GP, Weaver AL, Polis AB, Dixon ME, Schnitzer TJ (2002) Efficacy of rofecoxib, celecoxib, and acetaminophen in osteoarthritis of the knee. *J Am Med Assoc* 287(1):64–71
5. Altman RD (1999) Ibuprofen, acetaminophen and placebo in osteoarthritis of the knee: a six-day double-blind study [abstract]. *Arthritis Rheum* 42:S403
6. Bresalier RS, Sandler RS, Quan H et al (2005) Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* 352:1092–1102
7. Solomon SD, McMurray JJ, Pfeffer MA et al (2005) Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 352:1071–1080
8. Nussmeier NA, Whelton AA, Brown MT et al (2005) Complications of the COX-2 inhibitors parecoxib and valdecoxib after cardiac surgery. *N Engl J Med* 352:1081–1091
9. Singh G, Wu O, Langhorne P, Madhok R (2006) Risk of acute myocardial infarction with nonselective non-steroidal anti-inflammatory drugs: a meta-analysis. *Arthritis Res Ther* 8(5):153–162
10. Deeks JD, Smith LA, Bradley MD (2002) Efficacy, tolerability, and upper gastrointestinal safety of celecoxib for treatment of osteoarthritis and rheumatoid arthritis: systematic review of randomised controlled trials. *Br Med J* 325:619–627
11. Laine L (1996) Nonsteroidal anti-inflammatory drug gastropathy. *Gastrointest Endosc Clin N Am* 6(3):489–504
12. Clegg DO, Reda DJ, Harris CL et al (2006) Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. *N Engl J Med* 354(8):795–808
13. Herrero-Beaumont G, Ivorra JAR, Trabado MC et al (2007) Glucosamine sulfate in the treatment of knee osteoarthritis symptoms. *Arthritis Rheum* 56(2):555–567
14. U.N. Food & Agriculture Organization: *FAO Statistical Yearbook—United States of America* (2004)
15. Wong M, Hendrix MJC, von der Mark K et al (1984) Collagen in the egg shell membranes of the hen. *Devel Biol* 104(1):28–36
16. Baker JR, Balch DA (1962) A study of the organic material of hen's-egg shell. *Biochem J* 82:352–361
17. Picard J, Paul-Gardais A, Vedel M (1973) Sulfated glycoproteins from egg shell membranes and hen oviduct. Isolation and

- characterization of sulfated glycopeptides. *Biochimica et Biophysica Acta* 320:427–441
18. Long FD, Adams RG, and DeVore DP (September 20, 2005) Preparation of hyaluronic acid from eggshell membrane. USA Patent #6946551
 19. Nakano T, Ikawa NI, Ozimek L (2003) Chemical composition of chicken eggshell and shell membranes. *Poult Sci* 82:510–514
 20. Starcher BC, King GS (1980) The presence of desmosine and isodesmosine in eggshell membrane protein. *Connect Tissue Res* 8:53–55
 21. Gautron J, Hincke MT, Panheleux M et al (2001) Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. *Conn Tissue Res* 42:255–267
 22. Akagawa M, Wako Y, Suyama K (1999) The presence of desmosine and isodesmosine in eggshell membrane protein. *Biochim Biophys Acta* 14:151–160
 23. Hincke MT, Gautron J, Panheleux M et al (2000) Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. *Matrix Biol* 19:443–453
 24. Hochberg MC, Chang RW, Dwosh I, Lindsey S, Pincus T, Wolfe F (1992) The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis. *Arthritis Rheum* 35:498–502
 25. Bellamy N, Buchanan WW, Goldsmith CH, Campbell J, Stitt LW (1988) Validation study of WOMAC: a health status instrument for measuring clinically important patient relevant outcomes to antirheumatic drug therapy in patients with osteoarthritis of the hip or knee. *J Rheumatol* 15:1833–1840
 26. Pham T, van der Heijde D, Altman RD et al (2004) OMERACT-OARSI initiative: Osteoarthritis Research Society International set of responder criteria for osteoarthritis clinical trials revisited. *Osteoarthritis Cartilage* 12:389–399
 27. Systat Software, Inc. , San Jose, CA, USA: www.systat.com
 28. Tubach F, Ravaud P, Giraudeau B (2005) Managing osteoarthritis of the knee: conclusions about use of NSAIDs are misleading. *Br Med J* 330:672
 29. McQuay H, Moore A (2007) Utility of clinical trial results for clinical practice. *Eur J Pain* 11:123–124
 30. Moore RA, Moore OA, Derry S, McQuay HJ (2008) Numbers needed to treat calculated from responder rates give a better indication of efficacy in osteoarthritis trials than mean pain scores. *Arthritis Res Ther* 10(2):5
 31. Lötsch J, Geisslinger G (2006) Current evidence for a genetic modulation of the response to analgesics. *Pain* 121:1–5
 32. Saver JL (2004) Number needed to treat estimates incorporating effects over the entire range of clinical outcomes. *Arch Neurol* 61:1066–1071
 33. Wen L, Badgett R, Cornell J (2005) Number needed to treat: a descriptor for weighing therapeutic options. *Am J Health Syst Pharm* 62(19):2031–2036
 34. Kristensen LE, Christensen R, Bliddal H, Geborek P, Danneskiold-Samsøe B, Saxne T (2007) The number needed to treat for adalimumab, etanercept, and infliximab based on ACR50 response in three randomized controlled trials on established rheumatoid arthritis: a systematic literature review. *Scand J Rheumatol* 36:411–417

Key Messages

Natural Eggshell Membrane (NEM®), 500 mg taken once daily, significantly reduced both joint pain and stiffness compared to placebo, both rapidly (10 days) and continuously (60 days).

Eggshell membrane: A possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies

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Background: Natural Eggshell Membrane (NEM®) is a novel dietary supplement that contains naturally occurring glycosaminoglycans and proteins essential for maintaining healthy joint and connective tissues. Two single center, open-label human clinical studies were conducted to evaluate the efficacy and safety of NEM® as a treatment for pain and inflexibility associated with joint and connective tissue disorders.

Methods: Eleven (single-arm trial) and 28 (double-arm trial) patients received oral NEM® 500 mg once daily for four weeks. The primary outcome measure was to evaluate the change in general pain associated with the treatment joints/areas (both studies). In the single-arm trial, range of motion (ROM) and related ROM-associated pain was also evaluated. The primary treatment response endpoints were at seven and 30 days. Both clinical assessments were performed on the intent-to-treat (ITT) population within each study.

Results: Single-arm trial: Supplementation with NEM® produced a significant treatment response at seven days for flexibility (27.8% increase; $P = 0.038$) and at 30 days for general pain (72.5% reduction; $P = 0.007$), flexibility (43.7% increase; $P = 0.006$), and ROM-associated pain (75.9% reduction; $P = 0.021$). Double-arm trial: Supplementation with NEM® produced a significant treatment response for pain at seven days for both treatment arms (X: 18.4% reduction; $P = 0.021$. Y: 31.3% reduction; $P = 0.014$). There was no clinically meaningful difference between treatment arms at seven days, so the Y arm crossed over to the X formulation for the remainder of the study. The significant treatment response continued through 30 days for pain (30.2% reduction; $P = 0.0001$). There were no adverse events reported during either study and the treatment was reported to be well tolerated by study participants.

Conclusions: Natural Eggshell Membrane (NEM®) is a possible new effective and safe therapeutic option for the treatment of pain and inflexibility associated with joint and connective tissue (JCT) disorders. Supplementation with NEM®, 500 mg taken once daily, significantly reduced pain, both rapidly (seven days) and continuously (30 days). It also showed clinically meaningful results from a brief responder analysis, demonstrating that significant proportions of treated patients may be helped considerably from NEM® supplementation. The Clinical Trial Registration numbers for these trials are: NCT00750230 and NCT00750854.

Keywords: arthritis, pain, stiffness, eggshell membrane, joint, connective tissue, complimentary, alternative

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Background

It is estimated that 140 million adults in the United States (US) suffer from some form of joint or connective tissue (JCT) disorder (ie, arthritis, lupus, gout, fibromyalgia, neck

or back pain, etc.).^{1,2} As the population ages, this estimate is expected to grow rapidly. Traditional treatments for most of these disorders attempt to address only the symptoms (pain, inflammation, and discomfort) associated with the diseases. This usually involves the use of analgesics (ie, acetaminophen, oxycodone, propoxyphene) or nonsteroidal anti-inflammatory drugs (NSAIDs) (ie, ibuprofen, diclofenac, celecoxib) alone or in combination. Most of these treatments have shown limited effectiveness in randomized controlled clinical trials (RCTs)^{3–6} or are known to have significant and sometimes severe side effects. To avoid the cardiac risks,^{7,8} gastrointestinal issues,^{9,10} and dependency issues^{11,12} associated with traditional JCT treatments (particularly with long-term use), many patients have turned to complementary and alternative medicines (CAMs) such as dietary supplements.

Glucosamine, chondroitin, and methylsulfonylmethane (MSM) alone and in combination, are widely marketed as dietary supplements to treat joint pain due to osteoarthritis (OA). There have been two major human clinical trials that have investigated the role of glucosamine and chondroitin in the treatment of OA symptoms. The Glucosamine/chondroitin Arthritis Intervention Trial (GAIT), a 1583 patient, six-month trial sponsored by the National Institutes of Health (NIH), failed to show significant improvement in the Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index in the overall patient population for glucosamine, chondroitin, or their combination.¹³ The Glucosamine Unum In Die (once-a-day) Efficacy (GUIDE) trial, a 318 patient, six-month European trial sponsored by industry, showed a small, 5%–6% improvement in total WOMAC Index score over placebo for glucosamine sulfate.¹⁴ Other vitamins, minerals, and botanicals such as kava, pine bark extract, capsicum, boswellia root extract, turmeric/curcumin, etc., are also marketed for various JCT pain maladies often with little or no clinical support. Because of their limited effectiveness, the search for additional CAMs to treat JCT disorders continues.

We present here the use of eggshell membrane as a possible new natural therapeutic for JCT disorders. Eggshell membrane is primarily composed of fibrous proteins such as collagen type I.¹⁵ However, eggshell membranes have also been shown to contain glycosaminoglycans, such as dermatan sulfate and chondroitin sulfate¹⁶ and sulfated glycoproteins including hexosamines, such as glucosamine.¹⁷ Other components identified in eggshell membranes are hyaluronic acid,¹⁸ sialic acid,¹⁹ desmosine and isodesmosine,²⁰ ovotransferrin,²¹ lysyl oxidase,²² lysozyme,²³ and β -N-acetylglucosaminidase.²⁴ The discovery of eggshell membrane as a natural source of combined collagen, glucosamine, chondroitin, and hyaluronic

acid has prompted the evaluation of this material as a potential treatment for joint and connective tissue pain. ESM Technologies, LLC (Carthage, MO, USA) has developed methods to efficiently and effectively separate eggshell membrane from eggshells to create an essentially shell-free eggshell membrane. The isolated membrane is then partially hydrolyzed using a proprietary process and dry-blended to produce 100% pure Natural Eggshell Membrane (NEM[®]) powder. Compositional analysis of NEM[®] conducted by ESM has identified a high content of protein and moderate quantities of glucosamine (up to 1% by dry weight), chondroitin sulfate (up to 2%), hyaluronic acid (up to 2%), and collagen (Type I, up to 25%).

Two 1-month pilot clinical studies were conducted to evaluate the efficacy and safety of NEM[®] for the relief of the pain and discomfort associated with joint and connective tissue disorders. The results are presented herein.

Methods

Study designs

Both studies were conducted according to a single center, open-label, controlled design and were conducted in the US in accordance with the US Food and Drug Administration's principles of Good Clinical Practice (Title 21, Code of Federal Regulations, Parts 50 and 56 and ICH E6) and the Declaration of Helsinki. The study protocols were approved by an independent institutional review board (The Copernicus Group) and patients provided their written informed consent to participate. Subjects were required to suspend all current pain relief medications in order to participate in the study. For the double-arm trial, eligible subjects were then randomized either to NEM[®] formulation X or NEM[®] formulation Y treatment groups in the order in which they were enrolled in the study using a block randomization table. NEM[®] formulations X and Y differed in the degree ($Y \sim 2.5 \times > X$) to which they were partially hydrolyzed prior to final ingredient blending. The clinical investigators were not blinded to treatment (open label) (both studies) or randomization (double-arm trial), however the subjects were blinded to randomization in the double-arm trial. Treatment consisted once daily orally of either NEM[®] (Membrell, LLC, Carthage, MO, USA) (single-arm trial) or two different formulations of NEM[®] (double-arm trial) provided in 500 mg vegetarian capsules that were stored in closed containers at ambient temperature. Clinic visits were scheduled for subjects at seven and 30 days following the onset of treatment. Treatment compliance was checked at clinic visits by patient interview and by counting the number of unused doses of the study medications. Although discouraged, acetaminophen was allowed for pain

relief rescue, if necessary. Subjects recorded the time and amount of acetaminophen taken in patient diaries.

Patients

All subjects aged 18 years or older and seeking relief of mild to moderate pain due to joint or connective tissue disorders were considered for enrollment in the studies. In order to be eligible, subjects must have had persistent pain (lasting at least three months) associated with a JCT disorder, with a minimum baseline pain level of 2 on a 0 to 10 analog Likert scale. Subjects were required to suspend all current pain relief medications. Subjects that were currently taking analgesic medications were eligible to participate in the studies following a 14-day washout period for NSAIDs, a seven-day washout for narcotics, and a 90-day washout for injected steroids. Subjects currently taking glucosamine, chondroitin sulfate or MSM were only eligible after a three-month washout period. Patients were excluded if they were currently receiving remission-inducing drugs such as methotrexate or immunosuppressive medications or had received them within the past three months. Other exclusionary criteria were: body weight 113.5 kilograms (250 lbs.) or greater, a known allergy to eggs or egg products, or pregnant or breastfeeding women. Subjects previously enrolled in a study to evaluate pain relief within the past six months or currently involved in any other research study involving an investigational product (drug, device, or biologic) or a new application of an approved product, within 30 days of screening were also excluded from participating in the trials.

Treatment response

Single-arm trial

The primary outcome measure of this study was to evaluate the mean effectiveness of NEM® in relieving general pain associated with moderate JCT disorders. Additional primary outcome measures were to evaluate flexibility, as well as the pain associated with the range of motion (ROM) evaluation. The primary treatment response endpoints were the 7 and 30 day clinic assessments utilizing a 0 to 10 analog Likert-scale, with 0 equating to no pain and 10 equating to most severe pain. Patients were asked to record a number equating to the perceived pain from the treatment joint/area. Endpoints were then compared to pretreatment assessments.

Double-arm trial

The primary outcome measure of this study was to evaluate the mean effectiveness of NEM® in relieving general pain associated with moderate JCT disorders. Subjects were allowed to evaluate multiple treatment joints/areas. The primary treatment response endpoints were the seven and 30 day

clinic assessments utilizing a 0 to 10 analog Likert scale, with 0 equating to no pain and 10 equating to most severe pain. Patients were asked to record a number equating to the perceived pain from the treatment joints/areas. Endpoints were then compared to pretreatment assessments.

Adverse events

Secondary objectives of both studies were to evaluate tolerability and any adverse reactions associated with supplementation with NEM®. The subjects' self-assessment diaries were reviewed and any discomfort or other adverse events were recorded and reported in accordance with applicable FDA regulations. Adverse events and serious adverse events were assessed by the clinical investigator at each study visit and followed until resolution, as necessary. Serious adverse events (those that result in either death, a life-threatening experience, inpatient hospitalization, a persistent or significant disability or incapacity; or require, based on reasonable medical judgment, a medical or surgical intervention to prevent an outcome described previously) were required to be reported to the clinical monitor immediately.

Statistical analysis

As these were pilot studies with nonspecified treatment joints/areas, no enrollment sample size estimates were performed for statistical power determination. Comparisons of the two treatment groups (double-arm trial) were made with a two-sided independent group *t*-test at baseline to validate randomization. Statistical significance was accepted at an α value of <0.05 . Post-baseline statistical analyses were done as pairwise comparisons utilizing a two-sided *t*-test. Statistical significance was accepted at an α value of <0.05 . Analysis of the primary outcome measure (the change from baseline in general pain levels (both studies), as well as flexibility and ROM-associated pain (single-arm trial)) was conducted in the intent-to-treat (ITT) population (ie, including all patients with at least one efficacy assessment). The last observation carried forward (LOCF) approach was used for patients who made at least one follow-up visit but who did not complete the study (lost to follow-up). A per-protocol completer analysis was also performed. SYSTAT software (version 12) was used for all statistical analyses.²⁵

Results

Single-arm trial

Patient recruitment began in October 2003 at a single clinical site in Missouri (USA) and the final follow-up was conducted in December 2003. A total of 11 subjects were enrolled with

various joint and connective tissue conditions. Of these subjects, five (45%) were female and six (55%) were male. The treatment joints/areas consisted of knees (3), hips (1), elbows (1), neck (1), shoulders (1), and lower back (4). All 11 subjects completed baseline evaluations and were therefore used as the ITT population. Ten (91%) of the 11 ITT subjects completed the one-month study per the protocol. Of the 11 subjects in the ITT population, one subject did not begin treatment and, therefore, was not available for further analysis. Compliance with the study treatment regimen was good in the treatment group. In those subjects that completed the study, the rate of compliance was >98% (as judged by capsule count at clinic visits).

A clinical comparison of valid subjects was carried out to obtain a mean baseline pain value for the study population of 3.6 ± 1.8 (mean \pm standard deviation), a mean flexion ROM of $64.2^\circ \pm 36.5^\circ$, and a mean ROM-associated pain value of 2.9 ± 2.6 . Statistical analysis of the primary outcome measures revealed that supplementation with NEM[®] produced a significant treatment response at seven days for flexibility (27.8% increase; $P = 0.038$) and at 30 days for general pain (72.5% reduction; $P = 0.007$), flexibility (43.7% increase; $P = 0.006$), and ROM-associated pain (75.9% reduction; $P = 0.021$) (see Table 1). Overall, the use of rescue pain medication was extremely low throughout the study, occurring once in every 20–22 days.

Double-arm trial

Patient recruitment began in November 2003 at a single clinical site in Missouri (USA) and the final follow-up was conducted in February 2004. A total of 28 subjects were enrolled with various joint and connective tissue conditions, some with multiple treatment joints/areas. Of these subjects, six (21%) were male and 22 (79%) were female. The treatment joints/areas consisted of knees (7), hips (8), neck (1), shoulders (9), hands (2), legs (1), feet (1), lower back (4), and nonspecific (3). All 28 subjects completed baseline evaluations and were therefore used as the ITT population. Fourteen subjects (50%) were randomized to the NEM[®] X formulation treatment group and 14 subjects (50%) were randomized to the NEM[®] Y formulation treatment group. Twenty (71%) of the 28 ITT subjects completed the one-month study per the protocol. Of the 28 subjects in the ITT population, two subjects assigned to the X arm did not begin treatment and, therefore, were not available for further analysis. One patient in the X arm and 1 patient in the Y arm officially withdrew from the study due to lack of efficacy. The overall drop-out rate of 29% (from enrollment) was higher than expected, although 77% of the subjects that began treatment ($n = 26$) completed the study. Compliance with the study

treatment regimen was good in both treatment groups. In those subjects that completed the study, the rate of compliance was >96% (as judged by capsule count at clinic visits).

Patient data was initially evaluated to ensure randomization between groups ($P = 0.097$). A clinical comparison of valid subjects was carried out to obtain a mean baseline pain value (mean \pm standard deviation) for each arm (X and Y) of the study (X: 6.8 ± 1.9 ; Y: 5.6 ± 1.9). Statistical analysis of the primary outcome measures revealed that supplementation with NEM[®] produced a significant rapid treatment response for pain at seven days for both treatment arms (X: 18.4% reduction; $P = 0.021$. Y: 31.3% reduction; $P = 0.014$). The 12.9% difference between treatment arms was not as large as was expected from the difference in the formulations. It was decided that this difference was not clinically meaningful. Therefore, at seven days the Y arm crossed over to the X formulation for the remainder of the study. The significant treatment response continued through 30 days for pain (30.2% reduction; $P = 0.0001$) (See Table 2). Overall, the use of rescue pain medication was extremely low throughout the study, occurring once in every 14–16 days. There were no significant differences between the treatment arms for rescue medication use.

Both study populations were too small to stratify the patients according to covariates, such as gender or treatment joint/area, to obtain statistically relevant data. There were no adverse events or serious adverse events reported during either of the studies. Of particular note is that there were no allergy-associated adverse events during the studies, although those with known egg allergies were excluded from participating during screening. In general, the treatment was reported to be extremely well tolerated by study participants.

Discussion

Joint and connective tissue disorders are extremely common in the United States and result in significant costs, both financial and quality of life, for those that suffer from the debilitating diseases. These two pilot clinical trials were designed to evaluate the efficacy and safety of NEM[®] as a treatment option for JCT disorders. Results from both pilot studies suggest that NEM[®] is both effective and safe for treating pain associated with JCT disorders and considerably improves flexibility in the affected joints/areas. NEM[®] has the added benefit of avoiding the concerning side effects associated with long-term use of other JCT treatments, such as narcotics or NSAIDs.

Patients experienced relatively rapid (seven days) responses for pain (double-arm trial) with a mean response of approximately 25% (X: 18.4%; Y: 31.3%) and flexibility (single-arm trial) with a mean response of approximately 28%.

Table 1 Single-arm trial mean values by category at baseline, 7 and 30 days post-treatment

	Days post-treatment	Mean \pm SD	Percent improvement	P-value
General Pain	Baseline (n = 11)	3.6 \pm 1.8	–	–
	7 (n = 11)	2.7 \pm 1.7	25.8%	0.515
	30 (n = 11)	1.0 \pm 1.2	72.5%	*0.007
Flexion (ROM)	Baseline (n = 11)	64.2° \pm 36.5°	–	–
	7 (n = 11)	82.0° \pm 41.4°	27.8%	*0.038
	30 (n = 11)	92.2° \pm 38.4°	43.7%	*0.006
ROM Pain	Baseline (n = 11)	2.9 \pm 2.6	–	–
	7 (n = 11)	1.7 \pm 2.1	43.3%	0.112
	30 (n = 11)	0.7 \pm 1.3	75.9%	*0.021

Notes: P-values were determined by pairwise, two-sided, t-test comparison, and represent treatment versus baseline. *P < 0.05.

Abbreviations: ROM, range of motion; SD, standard deviation.

The single-arm trial failed to reach statistical significance at seven days for pain. This is likely a result of the relatively high standard deviation at baseline (1.8) compared to mean pain at baseline (3.6). Each arm of the double-arm trial had a similar number of patients (X: 12; Y: 14) as in the single-arm trial (10) and yet had statistically significant results for pain at seven days. However the baseline means (X: 6.8; Y: 5.6) and standard deviations (both 1.9) were relatively better proportioned than the single-arm trial. The use of the pairwise t-test helps to mitigate the effects of a high standard deviation in determining statistical significance.

By the end of the follow-up period (30 days) the mean response for pain had improved to 30% (double-arm trial) and 73% (single-arm trial). At the same time, flexibility improved to a mean response of approximately 44% and the ROM-associated pain had a mean response of approximately 76% (single-arm trial). A brief responder analysis of the data provides a number of clinically relevant highlights. In both the single-arm trial and the double-arm trial, a significant proportion of the study populations (64% and 35%, respectively) experienced a greater than 50% reduction in pain by 30 days. Of particular note is that nearly half (45%) of the patients in the single-arm trial reported that they were pain-free (reported a score of 0) by 30 days of supplementation. All patients in the single-arm trial experienced at least some improvement in flexibility or

ROM-associated pain, with more than half (55%) of the subjects experiencing a greater than 50% improvement in flexibility and more than one-third (36%) of the subjects reporting that they were pain-free during ROM evaluation. The safety profile for NEM® is also of significance as there are no known side effects, excluding the obvious egg allergy concern. This is of obvious importance in a condition that requires long-term treatment such as JCT disorders. Significant and sometimes serious side effects associated with other treatments can force patients to have to make the difficult decision between living with the disease symptoms or living with the side effect symptoms.

Both trials suffered from a limited initial enrollment (11 and 28 subjects), however both trials had relatively low drop-out rates (9% and 29%). As both trials were also open label, there is the obvious issue of the placebo effect. The inclusion of a placebo control would have provided greater clinical meaning, however it would have required a significantly larger study population. The inclusion of such a broad range of JCT disorders with vastly differing pathologies could have readily led to ambiguous treatment effects. Additionally, a broad spectrum of disease severity was included in both trials which could have also led to ambiguous results. Despite these potential pitfalls, the results from these open-label trials suggest that NEM® may be an effective therapeutic for a broad range of JCT disorders. A larger study with some modifications is clearly warranted.

Table 2 Double-arm trial mean pain values at baseline, 7 and 30 days post-treatment

Days post-treatment	X Mean \pm SD	Y Mean \pm SD	Percent improvement	P-value
Baseline (n = 12, 14)	6.8 \pm 1.9	5.6 \pm 1.9	–	–
7 (n = 12, 14)	5.5 \pm 2.0	3.9 \pm 2.5	18.4%, 31.3%	*0.021, *0.014
30 (n = 26)	4.3 \pm 2.3	–	30.2%	*0.0001

Notes: P-values were determined by pairwise, two-sided, t-test comparison, and represent treatment versus baseline. *P < 0.05.

Abbreviation: SD, standard deviation.

Conclusion

With so many people suffering from joint and connective tissue disorders, and that number expected to grow immensely as the overall US population ages, it is important for patients to have treatment options that are both effective and safe. The reporting of the results from these two open-label pilot studies demonstrates that NEM[®] may be a viable treatment option for the management of JCT disorders. In these clinical studies, NEM[®], 500 mg taken once daily, significantly reduced pain, both rapidly (seven days) and continuously (30 days). It also showed clinically meaningful results from a brief responder analysis, demonstrating that a significant proportion of treated patients may benefit from NEM[®] supplementation.

Disclosure

KJR is currently employed by the sponsor of both studies. DPD has served as a paid consultant for the sponsor of the studies. MDL and MAR have no competing interests. KJR carried out the statistical analysis of the data and drafted the manuscript. DPD participated in the design of both studies, assisted in the statistical analysis of the data, and assisted in drafting the manuscript. MDL was the clinical investigator for the Double-Arm Trial and assisted in drafting the manuscript. MAR was the clinical investigator for the Single-Arm Trial and assisted in drafting the manuscript. All authors read and approved the final manuscript. The authors would like to acknowledge Dr Barry W. Ritz, Drexel University, Department of Bioscience and Biotechnology, USA, for his assistance with the preparation of this manuscript. Both studies were sponsored by ESM Technologies, LLC.

References

- Helmick CG, Felson DT, Lawrence RC, et al. Estimates of the Prevalence of Arthritis and Other Rheumatic Conditions in the United States. Part I. *Arthritis Rheum.* 2008;58(1):15–25.
- Lawrence RC, Felson DT, Helmick CG, et al. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. *Arthritis Rheum.* 2008;58(1):26–35.
- Altman RD. Ibuprofen, acetaminophen and placebo in osteoarthritis of the knee: a six-day double-blind study [abstract]. *Arthritis Rheum.* 1999;42:S403.
- Case JP, Baliunas AJ, Block JA. Lack of efficacy of acetaminophen in treating symptomatic knee osteoarthritis: A randomized, double-blind, placebo-controlled comparison trial with diclofenac sodium. *Arch Intern Med.* 2003;163:169–178.
- Geba GP, Weaver AL, Polis AB, Dixon ME, Schnitzer TJ. Efficacy of rofecoxib, celecoxib, and acetaminophen in osteoarthritis of the knee: a randomized trial. *JAMA.* 2002;287(1):64–71.
- Towheed TE, Maxwell L, Judd MG, Catton M, Hochberg MC, Wells G. Acetaminophen for osteoarthritis. *Cochrane Database Syst Rev.* 2006;1:CD004257.
- Singh G, Wu O, Langhorne P, Madhok R. Risk of acute myocardial infarction with nonselective non-steroidal anti-inflammatory drugs: a meta-analysis. *Arthritis Res Ther.* 2006;8(5):153–162.
- Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med.* 2005;352:1071–1080.
- Deeks JD, Smith LA, Bradley MD. Efficacy, tolerability, and upper gastrointestinal safety of celecoxib for treatment of osteoarthritis and rheumatoid arthritis: systematic review of randomised controlled trials. *Brit Med J.* 2002;325:619–627.
- Laine L. Nonsteroidal anti-inflammatory drug gastropathy. *Gastrointest Endosc Clin N Am.* 1996;6(3):489–504.
- Porter J, Jick H. Addiction rate in patients treated with narcotics. *N Engl J Med.* 1980;320:123–126.
- Weaver M, Schnoll S. Addiction issues in prescribing opioids for chronic nonmalignant pain. *J Addict Med.* 2007;1(1):2–10.
- Clegg DO, Reda DJ, Harris CL, et al. Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. *N Engl J Med.* 2006;354(8):795–808.
- Herrero-Beaumont G, Ivorra JAR, Trabado MC, et al. Glucosamine sulfate in the treatment of knee osteoarthritis symptoms. *Arthritis Rheum.* 2007;56(2):555–567.
- Wong M, Hendrix MJC, von der Mark K, Little C, Stern R. Collagen in the egg shell membranes of the hen. *Dev Biol.* 1984;104(1):28–36.
- Baker JR, Balch DA. A study of the organic material of hen's-egg shell. *Biochem J.* 1962;82:352–361.
- Picard J, Paul-Gardais A, Vedel M. Sulfated glycoproteins from egg shell membranes and hen oviduct. Isolation and characterization of sulfated glycopeptides. *Biochim Biophys Acta.* 1973;320:427–441.
- Long FD, Adams RG, DeVore DP. Preparation of hyaluronic acid from eggshell membrane. US Patent #6946551, September 20, 2005.
- Nakano T, Ikawa NI, Ozimek L. Chemical composition of chicken eggshell and shell membranes. *Poult Sci.* 2003;82:510–514.
- Starcher BC, King GS. The presence of desmosine and isodesmosine in eggshell membrane protein. *Connect Tissue Res.* 1980;8(1):53–55.
- Gautron J, Hincke MT, Panheleux M, et al. Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. *Conn Tissue Res.* 2001;42:255–267.
- Akagawa M, Wako Y, Suyama K. Lysyl oxidase coupled with catalase in egg shell membrane. *Biochim Biophys Acta.* 1999;1434(1):151–160.
- Hincke MT, Gautron J, Panheleux M, et al. Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. *Matrix Biol.* 2000;19:443–453.
- Ahlborn GJ, Clare DA, Sheldon BW, Kelly RW. Identification of eggshell membrane proteins and purification of ovotransferrin and β -NAGase from hen egg white. *Protein J.* 2006;25(1):71–81.
- Systat Software, Inc., San Jose, CA. 2007. Accessed May 10, 2007. Available from: <http://www.systat.com/>.

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