Safety Evaluation of NEM®:
An 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 hours. 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 5,000 μg/plate. NEM® did not exhibit any signs of acute toxicity in rats at a single oral dose of up to 2,000 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 2,000 mg/kg body weight per day for 90 days. The results of these studies suggest that NEM® may be safe for human consumption.

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. Eggshell membranes are an abundant raw material that are a novel source for naturally occurring bioactive compounds such as glucosamine (1), chondroitin sulfate (2), hyaluronic acid (3), collagen Type I (4), and sulfur-rich proteins (5). In the U.S. alone, an estimated 600,000 tons of eggshells are produced annually as a by-product of the egg products industry (6). Disposal of these eggshells creates an environmental and financial burden and, therefore, alternative uses for these materials is 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 (7).

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 (8; 9; 10; 11; 12). 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 (13). 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, genotoxicity, acute oral toxicity, and 90-day repeated dose oral toxicity. The results of these studies are presented herein.

Materials and Methods

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 (7). 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 three years from the date of manufacture.

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^{o}C (5% carbon dioxide and ≥90% humidity) for 1, 4.5, and 20 hours. 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 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.

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, 1,000, & 5,000 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, & 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, 1,000, & 5,000 μg/plate) of NEM® in both the presence 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 hours of incubation at 37^{o}C. Results of the initial assay were confirmed with a repeated assay using a 2.5X increase in S9 activator.

Animal Models of Toxicology

Acute oral toxicity in rats: Testing was performed by Pharmaceutical Control and Development Laboratory Co. Ltd. (Budapest, Hungary) following U.S. Food & 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 minutes 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-hour fasting period, the NEM® suspension was administered as a single dose at either 1,000 or 2,000 mg/kg body weight (bw) by oral gavage to 6-week-old Sprague Dawley (Crl:CD) rats (Charles River Laboratories Hungary Ltd. Isaszeg, Hungary) in groups of 10 (5 animals per sex per group, randomized by weight within an interval of ±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^{o}C; 30-70% humidity; 12-hour light/dark cycle) and had access to standard rat chow (Ssniff SM R/M-Z+H, Ssniff 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, or 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.

90-day oral toxicity in rats: Testing was performed by Ricerca Biosciences, LLC (Concord, OH USA) following U.S. Food & 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 mg/mL and 200 mg/mL, corresponding to a dose volume of 10 mL/kg. The test article was stored at approximately 4^{o}C with constant stirring between daily uses. The NEM® suspension was administered daily at doses of 0 (control, vehicle only), 400, or 2,000 mg/kg bw/day by oral gavage to 7-week-old Sprague Dawley (Crl:CD) rats (Charles River Laboratories Inc., Portage, MI USA) in groups of 10 (5 animals per sex per group, randomized by weight within an interval of ±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^{o}C; 30-70% humidity; 12-hour 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 hours 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 2,000 mg/kg test animals were preserved, fixed, and stained (hematoxylin & 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 (TBILI), 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).

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) (14) and Provantis software (version 8) (15) were used for statistical analyses.

Results

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 greater than twenty-four hours (>24 hrs), whereas Triton X-100 has an ET-50 of 2.3 hours.

Table 1: Percent of viable cells in NEM® and Triton X-100 (positive control) treated samples after various incubation periods.

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

Mutagenicity Evaluation

There were no significant revertant mutation rates observed exceeding the background average (spontaneous reversions) either with our 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 (50-5,000 μg/plate). The results of a repeated assay with 2.5X more S9 activator confirmed the negative results of the initial assay (data not shown).

Table 2: Revertant colonies per plate in control (0 μg/plate) and NEM® dosed plates (50-5,000 μg/plate), both with and without S9 metabolic activation.

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
1,000	33 ± 2	181 ± 11	23 ± 3	11 ± 3	86 ± 9	40 ± 7	179 ± 8	21 ± 1	10 ± 1	95 ± 12
5,000	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.

Animal Models of Toxicology

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 LD_{50} of greater than 2,000 mg/kg bw and indicates a low order of acute toxicity.

Figure 1: Mean daily food consumption during 90-day oral toxicological evaluation.

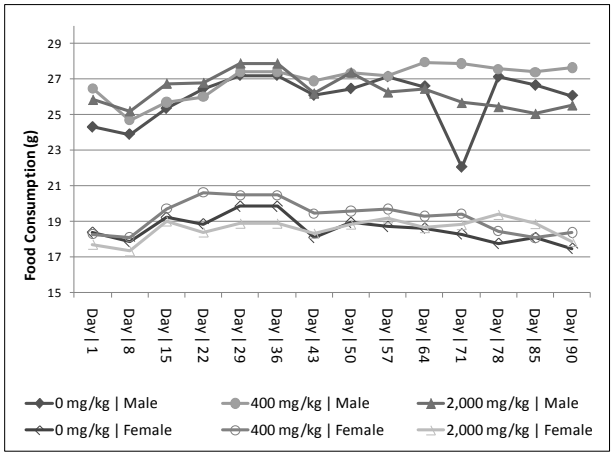
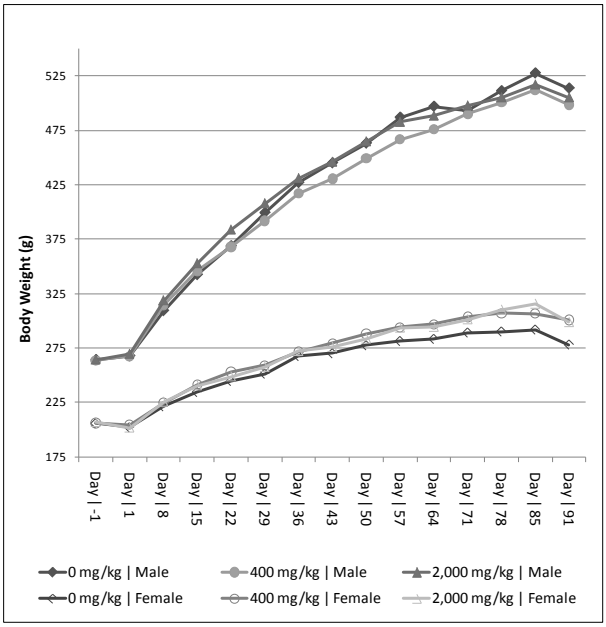


Figure 2: Mean body weight during 90-day oral toxicological evaluation.



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 (Figure 1) or body weight gain (Figure 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 2,000 mg/kg bw/day treatment group. Due to the possibility of a treatment-related effect, ovaries from the 2,000 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 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 considered to be greater than 2,000 mg/kg bw/day (the highest dose level administered).

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	2,000	0	400	2,000
Mean Organ Weights (g)						
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*	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*
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
Mean Organ Weight relative to body weight (g/kg)						
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*	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
Mean Organ Weight relative to brain weight (g/g)						
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*
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, * = $p < 0.05$, ** = $p < 0.01$

Table 4: Urinalysis parameters 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	2,000	0	400	2,000
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†	NEG	NEG	NEG
KET (mg/dL)	3.2 ± 6.6	0.2 ± 0.0	6.6 ± 7.7	NEG	NEG†	NEG†
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†	NEG	NEG	NEG†	NEG†
LEU	NEG†	NEG	NEG†	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 = leukocytes, BLD = occult blood, NEG = negative, † = 1 animal positive, * = $p < 0.05$, ** = $p < 0.01$

Table 5: Hematological parameters 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	2,000	0	400	2,000
RBC (M/uL)	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, * = $p < 0.05$, ** = $p < 0.01$

Table 6: Coagulation-related parameters 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	2,000	0	400	2,000
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*	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**	13.9 ± 0.6*

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

Table 7: Clinical chemistry parameters 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	2,000	0	400	2,000
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, * = $p < 0.05$, ** = $p < 0.01$

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	2,000	0	400	2,000
Adrenal Glands						
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
Heart						
Minimal mononuclear cell infiltration	1/5	-	3/5	0/5	-	1/5
Mild mononuclear cell infiltration	1/5	-	1/5	0/5	-	0/5
Kidneys						
Minimal chronic progressive nephropathy	2/5	-	2/5	2/5	-	1/5
Liver						
Minimal mononuclear cell infiltration	4/5	-	4/5	3/5	-	5/5
Lungs						
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
Ovaries						
Decreased corpora lutea	-	-	-	1/5	1/5	0/5
Pancreas						
Mild subacute interstitial inflammation	1/5	-	0/5	0/5	-	0/5
Thymus						
Dark red pinpoint discoloration	3/5	1/5	3/5	0/5	0/5	0/5

- = no data

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 & stiffness (16) and increasing flexibility (17) 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 (18), 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 indications 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 (50-5,000 μg/plate) with no indication of a dose-related mutagenic effect, including a repeated assay with 2.5X 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 2,000 mg/kg body weight without the observance 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 2,000 mg/kg bw/day. These dose levels correspond to approximately ten times (10X) and fifty times (50X) the human equivalent dose (HED) of 500 mg per day, respectively (18). 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.

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 the safety of this product for use in either 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.

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