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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.

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 Pakan, 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; TBILI, total bilirubin; TPRO, total protein; TRIG, triglycerides; UBILI, 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 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.

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

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.

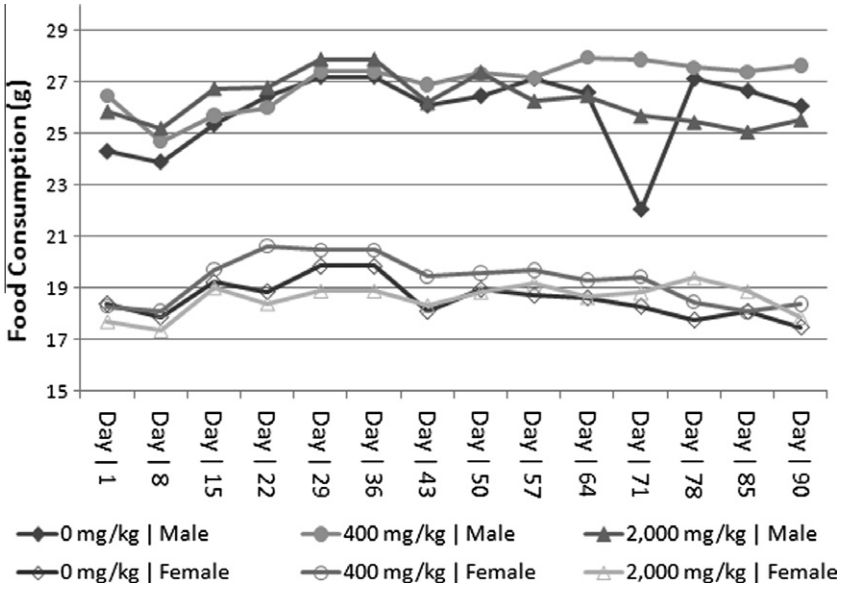

Fig. 1. Mean daily food consumption during 90-day oral toxicological evaluation of NEM® (0, 400, and 2000 mg/kg bw/day) in rats.