Safety assessment of Apoaequorin, a protein preparation: Subchronic toxicity study in rats

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A B S T R A C T

Apoaequorin, a calcium-binding protein originally isolated from jellyfish is available commercially as a dietary supplement. The objective of the present study was to investigate potential adverse effects, if any, of Apoaequorin, a recombinant protein preparation, in rats following subchronic administration. For this study, Sprague–Dawley (Hsd:SD) rats (10/sex/group) were administered via oral gavage 0 (control), 92.6, 462.9, and 926.0 mg/kg body weight (bw)/day of Apoaequorin preparation, for 90 days. The corresponding amount of Apoaequorin protein was 0, 66.7, 333.3 and 666.7 mg/kg bw/day, respectively. Administration of the Apoaequorin preparation did not result in any mortality. There were no clinical or ophthalmological signs, body weight, body weight gain, food consumption, food efficiency, clinical pathology or histopathological changes attributable to administration of Apoaequorin. Any changes noted were incidental and in agreement with those historically observed in the age and strain of rats used in this study. Based on the results of this study, the No Observed-Adverse-Effect Level (NOAEL) for Apoaequorin was determined as 666.7 mg/kg bw/day, the highest dose tested.

1. Introduction

Aequorin, a photoprotein is isolated from luminescent jellyfish (various Aequorea species, e.g., Aequorea victoria) and a variety of other marine organisms. This photoprotein is composed of two distinct units, the apoprotein, Apoaequorin and the prosthetic group coelenterazine, a luciferin (obtained from the animal’s diet). Aequorin emits blue light by an intramolecular reaction when mixed with Ca2+ (Shimomura et al., 1974). The chemical steps leading to light emission, involving the oxidative degradation of coelenterazine, have been extensively investigated (Inoue et al., 1989; Shimomura et al., 1993). For over four decades, the protein has been used as a bio-luminescent indicator to elucidate the role of calcium as a second messenger in cell signaling cascades (Kendall and Badminton, 1998). This has been accomplished by injecting the protein into living cells or more recently, developing recombinant expression cassettes to transiently transform cell types targeting of the protein to specific organelles (Vernon and Printen, 2002). In this case the prosthetic group, coelenterazine, is then added to the medium in which the cells grow to permit it to conjugate with the protein; forming the aequorin molecule. When cells are stimulated to release calcium ions the protein may bind the ion. This binding produces a photon of light. This light can be recorded in ways that allow the transient luminescence to indicate where calcium is being liberated inside the cell (Brini et al., 1995).

The protein, Apoaequorin, has been used in multiple cell types and has been localized to important intracellular compartments. Of the hundreds of published results using the photo-protein in this manner (Kendall and Badminton, 1998; Grienberger and Konnerth, 2012), none have indicated any deleterious or toxic effects on the cell types exposed to the protein. This is supported by the fact that the protein shares homologies with all EF-hand motifs for the super family of calcium-binding proteins of which there are several hundred in the human genome and over 400 predicted in the prokaryotic genome (Matsuo and Kanehisa, 1993; Zhou et al., 2006). The protein consists of 196 amino acid residues and is rich in all 20 essential amino acids making it a complete protein source. It is particularly rich in aromatic amino acids such as tryptophan (3% of the total amino acid composition), tyrosine (4%), and phenylalanine (4%).

The role of calcium in biology, including regulatory functions such as neuromuscular transmission, cellular secretions and blood clotting is widely recognized (Hendler and Rorvik, 2001). Alterations...
in calcium homeostasis have been claimed to underlie the reduced cellular function characteristic of the normal aging process of the brain (Foster and Norris, 1997; Kelly et al., 2006; Squier and Bigelow (2000); Thibault et al., 2007; Toescu and Vreugdenhil (2010); Verhratsky and Toescu, 1998). The endocrine system that helps maintain calcium homeostasis is integrated and complex. Calcium-binding proteins have been recognized as protective factors in neuronal populations susceptible to toxicity via calcium and calcium-mediated events (Alpár et al., 2012; Iacopino and Christakos, 1990; Foster et al., 2001; Kumar et al., 2009; Mattson et al., 2002; Rogers et al., 1990). The role of these proteins in the human body as protective agents have also been recognized (Heizmann and Braun, 1992). The available information indicate that calcium-channel blocking agents control the calcium ion from crossing calcium channels in order to reduce the signaling by the calcium ion and to reduce the adverse effects of calcium crossing through these channels.

In *in vitro* studies, Apoaequorin has been used to measure calcium response to extracellular stimuli in yeast cells (Nakajima-Shimada et al., 1991) and cell death following an ischemic insult (Choi, 1992). Detert et al. (2009) reported that direct hippocampal infusion of Apoaequorin in rats prior to oxygen and glucose deprivation showed protection of neurons from the ischemic insult. Preliminary clinical results indicate that daily consumption of Apoaequorin for 3 months may support improvements in recall in otherwise healthy adults (Underwood et al., 2011). These findings indicate possible beneficial neurologic effects of Apoaequorin protein. Given the potential use of Apoaequorin as a dietary supplement in possible support of cognitive health, toxicogenic potential of the protein was investigated in a long-term repeat dose toxicity study. In the repeat-dose toxicity study, a detailed assessment of the toxic potential of a well standardized Apoaequorin preparation when administered daily for 90-days via oral gavage to Sprague-Dawley rats was investigated.

2. Materials and methods

2.1. Study design

The study was performed in accordance with (A) FDA Redbook (2000): Chapter IV.C.4.a Subchronic Toxicity Studies with Rodents. (B) Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals, Section 4 Health Effects (Part 408); Repeated Dose 90-day Oral Toxicity Study in Rodents, and (C) standard operating procedures at Eurofins/Product Safety Labs (Dayton, NJ) and as per the mutually agreed study plan with the Sponsor. The study meets the requirements of 21 CFR 58: US FDA Good Laboratory Practices (GLP) Standards, 1987 and OECD Principles of Good Laboratory Practices (GLP), 1997 with the exceptions of serology and chemistry analysis that were not conducted in compliance with GLP regulations.

2.2. Test article

2.2.1. Neat test article

Standardized lyophilized Apoaequorin protein powder used in the present study was provided by Quincy Bioscience (Madison, WI, USA). The product is a tan colored freeze-dried, water-soluble powder, containing 722 mg active protein per gram dry powder as determined by RP-HPLC. The protein is produced through recombinant DNA technology using a non-toxic and non-pathogenic strain of *Escherichia coli*. The microorganism is allowed to ferment and the protein is recovered from the fermentation broth by extraction, purification, concentration and drying. Clarification of the fermentation mash through hollow fiber tangential flow filtration removes cells and results in a clear aqueous broth in which the protein is suspended. Purification of the cell-free broth is performed by Quincy Bioscience (QB) using multiple batch-wise anion exchange column chromatography steps. The purified protein is then concentrated and freeze-dried and tested for potency and microbiology. The manufacturing process complies with the Good Manufacturing Practices (GMPs) for dietary supplement (21 CFR 111).

Apoaequorin is composed of 196 amino acids. The complete protein sequence is known and it contains all 20 amino acids commonly found in proteins. The physical and chemical specifications of the food grade product, lot number 1105149, have been fully developed (Table 1). The Apoaequorin protein powder is standardized to contain approximately 50–75% apoaequorin protein, 25–50% sodium chloride, and minor components of the purification buffer such as acetic acid (<7%). Reversed-phase HPLC is used to both quantitate and identify Apoaequorin in the final product, using a protein standard developed for this purpose and qualified through rigorous purification and test methods. In addition to HPLC, SDS-PAGE was also carried out to confirm the identity of the protein. The molecular weight of Apoaequorin (CAS No. 845410-67-5) is 22.3 kDa. Other specification parameters such as moisture and microbiological analysis for food borne pathogens are routinely monitored within specifications acceptable for dietary ingredients. The results of an in *vitro* study (Goodman and Onof-anti, 2010), in which Apoaequorin protein was subjected to proteolytic digestion in simulated gastric fluid (SGF) using pepsin enzyme (a digestive protease produced in the stomach) from porcine shows that the protein is digested or enzymatically hydrolyzed to individual amino acids that are likely to be absorbed in the digestive tract.

2.2.2. Neat test and dose formulation analysis

Apoaequorin, neat test article and in the dosing solutions were analyzed by RP-HPLC for stability, homogeneity and concentration verification as part of this 90 day study.

<table>
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2.3. Animals

Male and female Sprague–Dawley rats (Harlan, Indianapolis, IN) were used for the present study. Prior to initiation of testing, the rats were acclimatized to laboratory conditions for eight days. Following acclimatization, animals (n=80) were selected for the study on the basis of adequate body weight gain, freedom from clinical signs of disease or injury, and a body weight within ±20% of the mean within a sex. Selected rats were randomly distributed, by body weight using a stratified randomization procedure. The rats were equally divided into four groups (10/sex/group). The animals weighed 203–223 grams (males) and 169–194 grams (females) and were approximately 8 weeks of age at initiation of dosing. One group was used for vehicle control while the other three were used as treatment groups and received the test substance, Apoaequorin protein.

The animals were individually housed in suspended stainless steel caging with mesh floors. Litter paper was placed beneath the cages and was changed at least three times per week. All animals were housed under standard laboratory conditions with adequate fresh air exchange, room temperature 19–25°C, relative humidity 40–80%, with 12 h light and 12 h dark cycle and the room was kept clean and vermin free. The animals were fed (ad libitum) with 2016CM Harlan Teklad Global Rodent Diet® throughout the acclimatization and experimental period. All animals were provided with filtered tap water (ad libitum) from an automatic water dispensing system.

2.4. Treatment

Rats, divided into four groups (10/sex/group), were treated orally (gavage) once daily with test substance preparation in deionized, reverse osmosis water (vehicle) at dose levels of 0 (Group 1 – control), 92.6 (Group 2 – low dose), 462.9 (Group 3 – mid dose), or 926.0 (Group 4 – high dose) mg/kg bw (dosing volume 10 mL/kg) for at least 90 consecutive days. The active ingredient (Apoaequorin protein) content of the test substance was 72%. Thus the equivalent dose of Apoaequorin protein received by each group was 0, 66.7, 333.3, and 666.7 mg/kg bw/day, respectively. Fresh aliquots containing 0.926% (low dose), 4.63% (intermediate dose), and 9.26% (high dose) of the test substance were prepared daily before administration. The test article was administered through oral route via gavage. The control group received the vehicle only at the same volume as the test animals. The dose levels selected were based on the expected human dose of approximately 20 mg/person/day (i.e. assuming a 60 kg human). The highest dose administered to animals was determined as 2000-times higher than a human recommended dosage of 20 mg protein/60 kg bw. This level was selected to achieve maximum administration of the protein at the highest volume humanly permitted (10 mL/kg per animal) and keep the protein in a soluble form. The oral (gavage) route was chosen to simulate the human exposure to this protein as a dietary supplement. The selected dose levels targeted approximately 200, 1000, and 2000 times the expected human oral intake on a 20 mg/day basis. Lower levels were determined at one-half and one-tenth of this maximum dose to allow for determination of an appropriate dose–response. Based on stability, homogeneity, and the concentration verification results of the test substance, the animals are considered to have received the targeted dose concentrations of Apoaequorin.

2.5. Parameters investigated

2.5.1. Clinical examination, body weight and feed consumption

All animals were observed once daily for clinical signs of toxicity and twice daily for mortality. On Day 1 (prior to first treatment with the test article) and approximately weekly thereafter, a detailed observation was conducted while handling the animal, generally occurring on days that the animals were weighed and food consumption measurements taken. Potential signs noted included, but were not limited to: changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions, and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Likewise, changes in gait, posture, and response to handling, as well as the presence of clear or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling), or bizarre behavior (e.g., self-mutilation, walking backwards) were also recorded.

Ophthalmological examination was performed on all animals before the start of study and on Day 90 of the study. Individual body weights were recorded at least two times prior to randomization. Test animals were weighed on Day 1 (prior to study start) and approximately weekly thereafter (intervals of 7 days ±1). The fasted test animals were also weighed immediately prior to euthanasia in order to calculate organ to body weight ratios. Body weight gain was calculated for selected intervals and for the study overall. Individual animal feed consumption was measured and recorded weekly to coincide with body weight measurements. Feed efficiency was calculated and reported. All animals were fasted overnight prior to blood collection.

2.5.2. Clinical pathology

Clinical pathology was performed on all animals for blood chemistry and hematology of the terminal sacrifice animals once toward the end of the in-life phase of the study. Blood samples for hematology (except coagulation samples) and clinical chemistry were collected via sublingual bleeding under isoflurane anesthesia on Day 86 of the test period. Approximately 0.5 mL was collected in a pre-calibrated tube containing 3KEDTA for hematological assessments. The whole blood samples were stored under refrigeration and shipped on cold packs. Approximately 1 mL was collected into a tube containing no preservative for clinical chemistry assessments. These samples were centrifuged in a refrigerated centrifuge and the serum was transferred to a labeled tube. Blood samples used to determine the prothrombin time and partial thromboplastin time (coagulation) were collected via the inferior vena cava under isoflurane anesthesia at termination (Day 93/94). All blood samples were evaluated for quality by visual examination. Upon completion of clinical chemistry, remaining serum samples from two animals were pooled for serology. The day before collection of samples for the clinical pathology evaluation, the animals were placed in metabolism cages. Animals were fasted after 3 PM (at least 15 h prior to) and urine was collected from each animal.

2.5.2.1. Hematology. Hematometry parameters included, erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), absolute reticulocyte count (ARET), platelet count (PLT), total white blood cell (WBC) and differential leukocyte count. Mean corpuscular hemoglobin concentration (MCHC) was calculated. In addition, separate, blood smears, stained with New Methylen Blue or Wright-Giemsa stain, were prepared from each animal undergoing hematological evaluation and were examined, if required, to substantiate or clarify the results of hematometry findings. Coagulation parameters included prothrombin time (PT) and active partial thromboplastin time (APTT).

2.5.2.2. Clinical chemistry. Clinical chemistry parameters measured included, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), alkaline phosphatase (ALKP), total bilirubin (BILI), urea nitrogen (BUN), blood creatinine (CREA), total cholesterol (CHOL), triglycerides (TRIG), fasting glucose (GLUC), total serum protein (TP), albumin (ALB), globulin (GLOB), calcium (CaLC), inorganic phosphorus (IPHS), sodium (NA), potassium (K), and chloride (Cl). Upon completion of clinical chemistry, remaining serum samples from two animals randomly selected animals were pooled for serology.

2.5.2.3. Urinalysis. Urinalysis parameters included quality, pH, ketone, color, glucose, bilirubin, clarity, specific gravity, blood volume, protein, urobilinogen, and microscopic urine sediment examination.

2.5.3. Pathology

2.5.3.1. Terminal necropsy and tissue collection. At termination all animals were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. All animals in the study were subjected to a full necropsy, which included examination of the external surface of the body, all orifices, and the thoracic, abdominal and cranial cavities and their contents. The following tissues were weighed as wet as soon as possible after dissection to avoid drying: adrenals (combined), kidneys (combined), spleen, brain, liver, thymus, epididymes (combined), ovaries (combined), uterus with oviducts, heart, and testes (combined). Tissues and organs procured, preserved in 10% neutral buffered formalin and processed for histopathological assessment included those according to documented guidelines. Additionally, epididymes, eyes with optic nerve and testes from all animals were preserved in modified Davidson's fixative and then stored in ethanol for histopathological examination. Additional tissues were preserved if indicated by signs of toxicity or target organ involvement.

2.5.3.2. Histopathological examination. The preserved organs and tissues of the animals from both the control and high dose groups (Groups 1 and 4, respectively) were subjected to histological examination. In addition, histological evaluations were performed on a spleen of a Group 3 male animal; uteri and oviducts in three Group 2 females and two Group 3 females; one stomach from a Group 2 female, and oviducts (female), adrenal glands (female) and small intestine (female) each in a Group 3 animal. The fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin and examined by light microscopy.

2.6. Statistical analysis

For the in-life and organ weight data, mean and standard deviations were calculated for all quantitative data. Data within groups were evaluated for homogeneity of variances and normality by Bartlett's test. Where Bartlett's test indicates homogeneous variances, treated and control groups were compared using a one-way analysis of variance (ANOVA). When one-way analysis of variance was significant, a comparison of the treated groups to control by Dunnett's test for multiple comparisons was performed. Where variances are considered significantly different by Bartlett's test, groups were compared using a nonparametric method (Kruskal-Wallis non-parametric analysis of variance). Where non-parametric analysis of variance was significant, comparison of treated groups to control was performed using Dunn’s test.
Clinical pathology data was initially evaluated by Levene’s test for homogeneity and Shapiro–Wilk test for normality. If preliminary test was not significant, one-way analysis of variance followed by Dunnett’s test was used, when significant, a one-way analysis of variance followed by Dunnett’s test was used. Significance was judged at a probability value of $p < 0.05$. Male and female rats were evaluated separately.

3. Results

3.1. Survival, clinical observations, ophthalmological examination

There were no test article-related or other mortalities during the course of study. There were no clinical signs in either male or female animals resulting from the administration of Apoaequorin. All clinical signs and observations were considered incidental and largely resolved by the end of the treatment phase (data not shown). Ophthalmoscopy prior to study initiation and near experimental completion (Day 90) did not reveal any abnormalities. However, during daily observations, two test-treated females one each in Groups 2 and 4 were noted as having the left eye partially closed. The confirmed blepharospasm was not associated with any conjunctival hyperemia or chemosis, nor was any intraocular abnormalities noted. The blepharospasm was attributed to environmental irritation, a common cause of surface ocular irritation in laboratory animal species. These findings were not attributed to exposure of the test substance, thus the test article was not considered an ocular toxicant.

3.2. Body weights and feed consumption

In male rats, the mean weekly body weights (Fig. 1) for the treated groups at 92.6, 462.9, and 926.0 mg/kg bw/day (Groups II – IV, respectively) were comparable with the control group values throughout the study. For all intervals other than those noted as statistically significant (Group IV increased, Days 1–8; Group III, decreased; Days 43–50, and Group IV decreased, Days 43–50), mean daily body weight gain (Table 2) for the treated male rats in Groups II – IV were generally comparable with the control group values throughout the study. Similarly, in females, the mean body weights for the treated female rats were comparable with the control group values throughout the study (Fig. 1). Except for statistically significant decreases in Group III during Days 64–71, mean daily body weight gain for the treated female rats were generally comparable with the control group values throughout the study (Fig. 1). Overall, there were no changes in body weight or body weight gain in male and female rats attributable to the administration of Apoaequorin. Any changes observed were sporadic and considered incidental, unassociated with test article administration.

Mean daily feed consumption for the treated male rats at 92.6, 462.9, and 926.0 mg/kg bw/day were comparable with the control group values throughout the study period (data not shown). For all intervals other than those noted as statistically significant (Groups III and IV, increased, Days 1–8, and on Days 43–50, decreased), mean feed efficiency for the treated male rats were generally comparable with the control group values throughout the study. Similarly, in females except for a statistically significant increase in Group III on Days 57–64, mean daily feed consumption for the treated female rats were generally comparable with the control group values throughout the study. A decrease in mean feed efficiency in Group III on Days 64–71 was noted. There were no changes in feed consumption or feed efficiency in male and female rats attributable to administration of Apoaequorin. Sporadic statistically significant changes in food consumption and feed efficiency were considered spurious, unassociated with test article administration.

3.3. Clinical pathology

3.3.1. Hematology

There were no treatment-related biologically significant effects of the Apoaequorin protein treatment at dose levels of 92.6, 462.9, and 926.0 mg/kg bw/day (Groups II – IV, respectively) in hematological parameters in male and female rats (Tables 3 and 4). Red cell distribution width in male rats administered 926.0 mg/kg bw/day of Apoaequorin (Group IV) showed a statistically significant increase. One female each in Groups II, and III was found to have absolute immature lymphocytes. Among the females of Group III, two revealed absolute atypical lymphocytes and one of these animals also exhibited absolute hypersegmented neutrophils; with no biologic relevance to treatment. Prothrombin time was decreased in males administered 92.6 and 926.0 mg/kg bw/day (Groups II and IV). These significant changes in mean hematology results were not observed in both sexes, lacked correlating changes in other clinical parameters, were of small magnitude, were not noted in a dose-related manner, or were not associated with microscopic changes in the related organs, and hence they were considered as incidental changes/biological variations and not treatment-related adverse effects. There were no test article-related changes in blood cell morphology.

3.3.2. Clinical chemistry

There were no treatment-related statistically significant effects of the Apoaequorin protein preparation at dose levels of 92.6,
462.9, and 926.0 mg/kg bw/day (Groups II, III and IV, respectively) in serum chemistry parameters in male and female rats (Tables 5 and 6). The serological analysis did not reveal any detectable titers against the usual battery of prospective pathogens and antigens tested.

### 3.3. Urinalysis

There were no treatment-related adverse effects in urinalysis parameters in male and female rats (data not shown) following administration of Apoaequorin protein preparation at dose levels of 92.6, 462.9, and 926.0 mg/kg bw/day (Groups II, III and IV, respectively).
respectively). The urinalysis parameters such as physical quality, pH, ketone, color, glucose, bilirubin, clarity, specific gravity, blood volume, protein, urobilinogen, and microscopic urine sediment examination did not reveal any statistical difference or physiological abnormalities in males and females in the control and treatment groups.

3.4. Organ weights

The changes noted in organ weights between the groups following treatment with Apoaequorin protein at dose levels of 92.6, 462.9, and 926.0 mg/kg bw/day for 90-days are summarized in Table 7. No toxicologically significant changes in organ weights were
observed in absolute (Table 7) and relative organ weights (data not shown) when compared to the vehicle control group. However, Group III female absolute heart weight and heart-to-brain weight ratio were statistically increased. This increase in heart weight was considered as incidental and toxicologically insignificant due to lack of dose-dependency and lack of correlating changes in clinical chemistry and histopathology.

3.5. Macroscopic findings

At scheduled necropsy there were no treatment-related gross pathological observations in any of the groups of rats following administration of the test article, Apoaequorin protein. Among the incidental changes noted included, the uterus/oviducts were noted as fluid-filled in 5 of 10 Group I animals at 0 mg/kg/day, in 4 of 10 Group II animals at 92.6 mg/kg/day, in 1 of 10 Group III animals at 462.9 mg/kg/day, and in 3 of 10 Group IV animals at 926.0 mg/kg/day of Apoaequorin. Microscopically, fluid-filled uteri usually corresponded microscopically to lumenal dilation of the uterus that was attributable to variation in the estrus cycle in individual animals, a finding not associated with test article administration.

The remaining macroscopic observations were of sporadic incidence and showed no trends/patterns to suggest a relationship to administration of Apoaequorin protein. These remaining macroscopic observations included: enlarged GALT (gut-associated lymphoid tissue) nodules, serosal side, involving the duodenum, jejunum, and ileum with Peyer’s patch (one female animal each
in Groups I, III, and IV) usually corresponding microscopically to slight to moderate lymphoid hyperplasia; a mass involving the oviduct (Group III – two females) that corresponded microscopically to single, unilateral cysts of the oviduct; and mottled adrenal glands, bilateral (Group III – one female) that was related to residual blood noted microscopically in adrenal gland sinusoids. Additionally, there were a few macroscopic observations noted that had no microscopic correlates. These included a red-brown stomach mass, 5 × 4 × 3 mm nodule on the serosal surface of the inner curvature of the stomach below esophageal junction in one female Group II rat, the tail of one spleen was slightly misshapen, tapered (Group III – one male), and a left ovary was observed to have a clear, firm cyst, (Group III – one female).

3.6. Microscopic findings

The incidence and severity of histopathological findings following treatment with Apoaequorin protein at dose levels of 92.6, 462.9, and 926.0 mg/kg bw/day for 90-days were summarized in Tables 8 and 9. There were no microscopic findings related to the administration of the test article, Apoaequorin. In the kidneys, there was minimal chronic progressive nephropathy (CPN) usually of minimal intensity in 9 of 10 males and 2 of 10 females of control group (0 mg/kg/day; Group I) as well as 8 of 10 males and 4 of 10 females of high dose group (926.0 mg/kg/day; Group IV). CPN was characterized by limited foci of tubular basophilia, thickened tubular basement membranes, tubular casts, and/or mononuclear cell infiltrates that affected less than 1% of nephrons. CPN at this low intensity with the observed incidence pattern was a background finding unrelated to the test article administration. As observed in this study, CPN is an incidental finding that occurs predominantly in males, occurs in Sprague–Dawley rats with increased incidence as compared to other rat strains, and can be seen histologically as early as two months of age (Percy and Barthold, 2007; Hard and Khan, 2004).

Additionally, there were a few microscopic observations of minimal to moderate intensity that were observed in single to a few males and/or females in Group IV that were not observed in males or females in Group I. These observations included chronic inflammation of the Harderian gland, coronary arteritis and cardiomyopathy, neutrophil infiltrates of the pancreas, ulcer of the forestomach, gastric squamous cyst, acute inflammation of the thyroid gland capsule (unilateral), and hepatocellular vacuolation. These limited findings were considered incidental and toxicologically irrelevant as there were no convincing incidence patterns/trends to suggest a relationship to test substance administration. The remaining microscopic findings were not test article-related and were incidental as can be observed in the age and strain of rats used in this study (Percy and Barthold, 2007).

4. Discussion

The results of present repeat-dose animal toxicity study show that oral (gavage) administration of recombinant Apoaequorin protein preparation at levels up to 926 mg/kg bw/day to male and female Sprague–Dawley rats for 90-days was not associated with adverse effects as evaluated by the general condition and appearance of the animals, growth, feed consumption, clinical observations, ophthalmoscopy, hematology, clinical chemistry, urinalysis, organ weights and histopathological findings. In general, treatment of the animals with Apoaequorin protein preparation at doses up to 926 mg/kg bw/day (equivalent to 666.7 mg Apoaequorin/kg bw/day) was well tolerated. The present sub-chronic study, statistically significant changes in some of the blood parameters in the Apoaequorin protein preparation treated groups were noted. For example, some of the hematological parameters such as red cell distribution width in male rats receiving high dose of Apoaequorin (926.0 mg/kg bw/day; Group IV) showed statistically significant increase. Additionally, one female rat from each of Group II (92.6 mg/kg bw/day) showed absolute immature lymphocytes. In Group III, one female showed absolute immature lymphocytes, two revealed absolute atypical lymphocytes and one of these animals also revealed absolute hypersegmented neutrophils. In Group II and Group IV male rats showed decrease in prothrombin time. The changes noted in hematology and clotting time in rats treated with Apoaequorin did not show any dose relationship and were noted in only one sex. Values for clotting time were within the historical control range as were those for platelets and calcium. In all cases, the magnitude of the changes was considered biologically insignificant. Hence, these changes were considered as incidental biological variations and not treatment-related.

Some limited histopathological findings such as chronic inflammation of the Harderian gland, coronary arteritis and cardiomyopathy, neutrophil infiltrates of the pancreas, ulcer of the forestomach, gastric squamous cyst, acute inflammation of the thyroid gland capsule (unilateral), and hepatocellular vacuolation.

### Table 8
Summary of histopathological findings from male rats treated with Apoaequorin protein preparation.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Lesion</th>
<th>mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(G-I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(G-II)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(G-III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(G-IV)</td>
</tr>
<tr>
<td>Epididymides</td>
<td>Cellular debris</td>
<td>1 ++</td>
</tr>
<tr>
<td>Hardian Glands</td>
<td>Chronic inflammation</td>
<td>1 ++</td>
</tr>
<tr>
<td>Heart</td>
<td>Arteritis</td>
<td>1 ++</td>
</tr>
<tr>
<td></td>
<td>Cardiomyopathy</td>
<td>1 *</td>
</tr>
<tr>
<td>Ileum</td>
<td>Hyperplasia, lymphoid</td>
<td>9 ++</td>
</tr>
<tr>
<td>Kidney</td>
<td>Chronic progressive nephropathy</td>
<td>8 ++</td>
</tr>
<tr>
<td>Liver</td>
<td>Mononuclear infiltrate</td>
<td>1 +</td>
</tr>
<tr>
<td></td>
<td>Vacuolization, hepatocyte</td>
<td>1 +</td>
</tr>
<tr>
<td>Nose and Nasal</td>
<td>Purulent exudate</td>
<td>1 ++</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Basophilic focus</td>
<td>1 ++</td>
</tr>
<tr>
<td></td>
<td>Neutrophil infiltrate</td>
<td>1 +</td>
</tr>
<tr>
<td>Parathyroid Glands</td>
<td>Ectopic thymus</td>
<td>1 ++</td>
</tr>
<tr>
<td>Pituitary Gland</td>
<td>Cyst</td>
<td>2 ++</td>
</tr>
<tr>
<td>Prostate</td>
<td>Chronic inflammation</td>
<td>4 ++</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Degeneration: myofiber</td>
<td>2 +</td>
</tr>
<tr>
<td>Testes</td>
<td>Atrophy: germ cell</td>
<td>1 ++</td>
</tr>
<tr>
<td>Urinary Bladder</td>
<td>Mononuclear infiltrate</td>
<td>1 +</td>
</tr>
</tbody>
</table>

* Grade 1 (minimal/very small/very few); ++ Grade 2 (slight/few/small); +++ Grade 3 (moderate-size/number).
were noted in the high dose group rats (Tables 8 and 9). However, as there were no convincing incidence patterns/trends to suggest a relationship to Apoaequorin treatment these changes were considered incidental and toxicologically irrelevant.

Apoaequorin is a small calcium-binding protein with properties typical of globular proteins having enzymatic function. It possesses features typical of EF-hand binding proteins (Head et al., 2000; Deng et al., 2005); being composed of amino acids important to human metabolism. Apoaequorin has been used for over 30 years in living systems as an analytical tool for cell biology without evidence of adverse biological effects. Apoaequorin shares homologies with calcium-binding proteins also found in human body (Matsuo and Kanesiha, 1993; Zhou et al., 2006) suggesting that it is unlikely to cause adverse effects. Apoaequorin, produced using recombinant technology, consists of 196 amino acid residues identical to naturally found aequorin protein from jellyfish. Historic account of human exposure to the edible jellyfish (Li and Hsieh, 2004; Kitamura and Omori, 2010), and in turn, exposure to the protein Apoaequorin, may give anecdotal evidence of its safety. In a series of assessments, Apoaequorin was tested for potential to cause allergenicity using Codex Alimentarius Guidelines such as in silico assessment of Apoaequorin amino acid sequence alignment to known allergens through an extensive bioinformatics, in vitro digestion and extensive literature search for any evidence of known allergenicity (Goodman, 2010). The results of these investigations did not suggest allergenic potential for Apoaequorin. The present study is conducted to provide a comprehensive assessment of the recombinant Apoaequorin protein according to accepted guidelines.

Further evidence of the safety of Apoaequorin is documented in a preliminary clinical trial (Underwood et al., 2011). In this double-blind study, a total of 218 healthy participants, aged 40 to 91 years (148 females; 70 males; 2 subjects lost) were randomly assigned to 4 groups based on weight to receive either Apoaequorin (10 mg) or placebo for three months duration. Participants were tested at 8, 30 and 90 day time points using quantitative, computer-based cognitive tasks of maze learning and recall (CogState Ltd.). The Apoaequorin arm showed a statistically significant decrease in the number of errors, and increase in accuracy of performance. In this 3 month study, Apoaequorin was well tolerated by the study participants with no reported adverse reactions (Underwood et al., 2011). Efficacy for the treatment of specific appropriate medical conditions must await further evaluation.

The results from present subchronic toxicity study showed no remarkable clinical, clinical pathology, or histopathology findings due to Apoaequorin treatment. Similarly, evaluation of the hematological parameters and the histopathological examination conducted in the present study revealed no evidence of any adverse effect systemically. The findings from this study suggest that oral administration of Apoaequorin at levels up to 666.7 mg/kg bw/day does not cause adverse effects in male and female rats. Based on the results of this study, the no-observed effect level (NOAEL) of Apoaequorin was 666.7 mg/kg bw/day, the highest dose tested. This NOAEL for Apoaequorin provides approximately 2000-fold safety factor over the recommended clinical dose of 20 mg/kg/day. Therefore, from the results of the study presented herein, it may be concluded that the use of appropriate levels of the recombinant protein Apoaequorin, as a dietary supplement is considered safe.

Conflict of Interest

Daniel L. Moran is employed by Quincy Bioscience. Madhu Soni works as an independent Consulting Toxicologist and assisted in the preparation of this manuscript.

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D.L. Moran et al. / Food and Chemical Toxicology 57 (2013) 1–10


