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ORIGINAL RESEARCH

Subchronic toxicity (90 days) of *StemEnhance*™ in Wistar rats

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Abstract: This study evaluated the subchronic toxicity of *StemEnhance*™, an extract of the blue-green alga *Aphanizomenon flos-aquae* that is used as a health supplement. Groups of 12 rats of each sex were given either 5% glycerin in water (control) or 200 mg/kg of *StemEnhance* prepared in 5% glycerin in water for 90 days by oral gavage. The administration of *StemEnhance* had no effect on behavior, food and water intake, growth, or survival. Values at the end of dosing and observation periods did not reveal differences between treated and control groups for hematology and clinical chemistry. There were no significant differences in the gross and histopathology of the reproductive organs in either males or females. Sperm motility parameters were similar for control and treated males. Our results show that *StemEnhance* at doses ~7 times the maximum label-recommended daily dose did not produce adverse effects in Wistar rats after subchronic treatment.

Keywords: algal toxicology, blue-green algae, cyanobacteria, *Aphanizomenon flos-aquae*

Introduction

Aphanizomenon flos-aquae (AFA) is a cyanobacterium ('blue-green algae', BGA) that grows worldwide. AFA is primarily harvested from Upper Klamath Lake in southern Oregon for use in nutraceuticals. AFA is a good source of dietary polyunsaturated fatty acids (PUFA). Dietary supplementation with 10% and 15% algae significantly decreased cholesterol and plasma triglyceride concentrations in rats.[1] Consumption of AFA (1.5 g) by 21 volunteers resulted in rapid changes in immune cell trafficking, but not direct activation of lymphocytes. That is, AFA increased immune surveillance without directly stimulating the immune system.[2]

AFA is a rich source of vitamins and minerals, antioxidant pigments, including C-phycocyanin and carotenoids, for example, β-carotene, flavacin, aphanin, and aphanicin in the epiphasic fraction and aphanizophyll in the hypophasic fraction.[3–6] Nearly 50% of the lipid content of dried AFA (5%–9% of total dry weight) is composed of dietary PUFA, mostly n-3 α-linolenic acid.[3]

StemEnhance™ is a blend (1 and 0.15 g, respectively) of two fractions extracted from AFA. The first extract (water or buffered saline), which contains an L-selectin ligand, supports the release of stem cells (CD34+ cells) from the bone marrow.[7] The other extract (10%–20% ethanol at 50°C), a polysaccharide-rich fraction named Migratose™, may support the migration of stem cells out of the blood into tissues. *StemEnhance* is commercially available as capsules for human consumption (recommended dose 1000–2000 mg/day). Toxicity studies of this plant extract are

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limited. National (eg, US Food and Drug Administration) and international organizations (eg, Organization for Economic Cooperation and Development (OECD)) require toxicological investigations, including short-term and long-term toxicity studies with rodents before certifying substances used for human consumption, prior to registering a product as generally recognized as safe (GRAS). Therefore, toxicological testing is needed in order to evaluate the safety of *StemEnhance*. The objective of this study was to evaluate the subchronic toxicological profile of *StemEnhance* in Wistar rats following daily administration of 200 mg/kg by oral gavage using a stomach tube for 90 days.

Materials and methods

Animals, housing, and test material

The protocol followed the OECD 408 (repeated dose 90-day oral toxicity study in rodents) guideline.[8] Wistar male and female rats (CRL:(WI)) ~7 weeks of age were used in this study (Charles River Laboratories, Inc, Wilmington, MA, USA). Forty-eight age and weight matched-rats were randomly allocated to the control and treatment groups (12 male and 12 female rats/group). Rats were housed individually in cages under standard laboratory conditions (temperature: 23 ± 2°C; relative humidity: 30%–70%; light–dark cycle: 12/12 h). The rats were acclimatized for 1 week in environmentally controlled cages to ensure that no abnormalities in each animal's condition existed prior to start of dosing.

Experimental design

The rats received a standard laboratory diet (Harlan 8604 rodent diet, Harlan Laboratories, Inc, Indianapolis, IN, USA) and tap water ad libitum. *StemEnhance* suspension was prepared as described in an earlier publication.[9] A constant volume of this suspension (100 mg/mL) (0.2 mL/100 g of body weight) was administered daily by oral gavage for 90 days. This dose is 7 times the maximum label-recommended daily dose for human consumption. The dose was given at a similar time each day and adjusted as necessary to maintain a constant dose level in terms of animal body weight. Control animals received an equal volume of water with 5% glycerin without *StemEnhance* using the same administration route. General clinical observations were made at least once a day, usually at the same time(s) each day. The health conditions of the animals were recorded. All animals were weighed once a week. Measurements of food and water consumptions were made at least weekly.

Gross pathology

Gross pathology was performed by a veterinary pathologist as described in an earlier publication.[9] Briefly, all of the animals were submitted to full necropsy procedures, including examination of the following organs and/or sites: external surface of the body; all orifices; cranial cavity; cervical tissues and organs; thoracic cavity and its contents, organs, and tissues; and abdominal cavity and its contents, organs, and tissues. Wet weight was determined for the following organs: liver, brain, spinal cord, heart, kidneys, spleen, adrenals, testes, epididymides, uterus, and ovaries as described in an earlier publication.[9] Organ weights were expressed as absolute values (g) and as relative (organ to body weights) values (gram per 100 g of body weight).

Histopathology

Full histopathology was carried out by a veterinary pathologist on all animals as described previously.[9] Briefly, harvested tissues were fixed in 10% neutral buffered formalin for a minimum of 48 h prior to trimming. Following fixation, tissues composed of bone were decalcified with a standard decalcifying solution (formic acid and sodium citrate). The fixed-decalcified tissues were exposed to routine processing, embedded in paraffin, and sectioned at 5 µm. Tissue sections were stained with hematoxylin and eosin using an automated staining system (Tissue Tek, Fisher Scientific, Waltham, MA, USA).

Hematology and clinical biochemistry

Blood samples were collected from the lateral vein of rats before dosing commenced. Additionally, at the end of the dosing period, blood samples were collected during necropsy directly from the heart for hematology (EDTA-containing microtubes were used as an anticoagulant) and clinical biochemistry analysis. Clotted and hemolyzed samples were excluded from analysis. Standard serum chemistry and hematology profiles were determined.[9] The following hematological examinations were made: packed cell volume (PCV), hemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leukocyte count (white blood cell (WBC)), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC). Additionally, the following clinical biochemical analyses of blood were carried out: glucose, calcium, phosphorus, creatinine, total protein, albumin, globulin, cholesterol, bicarbonate, anion gap, sodium, potassium, chloride, triglycerides, gamma-glutamyltransferase (GGT),

alkaline phosphatase (ALP), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and bilirubin. For hematology and serum chemistry parameters, Charles River Laboratory data was used as a reference range with the exception of the following serum chemistry parameters for which Boehm et al data was used; ALT, AST, calcium, cholesterol, creatinine, GGT, and triglycerides.[10,11]

Ki-67 immunohistochemistry

Ki-67 staining is a well-established marker of proliferative activity.[12] In the current study, the degree of uterine cellular proliferation in treated and control females was evaluated and compared with each other using Ki-67 immunohistochemistry (IHC) and image analysis IHC for Ki-67.[9]

Epididymal sperm count, motility, and morphology

At necropsy, the right cauda epididymis was immediately removed, weighed, and the tissue was processed.[9] Briefly, sperm counts were determined in six standard viewing fields and calculated per cauda and per gram of testis. A minimum of 200 sperms was analyzed per animal. The sperm motion parameters recorded were percentage of motile sperm, percentage of progressively motile sperm (progressive motility), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) beat cross frequency, straightness (STR), and linearity (LIN). Percentage progressive sperm is the ratio of the number of motile sperm with path velocity (VAP) $> V_0$ and STR $> S_0$ to the total number of sperm.[13]

For the sperm morphological analysis, 10 µL of the 1:9 diluted sperm from the motility analysis prepared as described previously[9] was pipetted on a pretreated glass microscope slide and spread with pipette tip to cover 1/2–2/3 of the slide. The slides were dried on a slide warmer at 37–40°C. The dried slides were placed in fixative (3 parts 100% ethanol and 1 part glacial acetic acid) for 2 min. The slides were drained and dried on a slide warmer. Fixed and dried sperm slides were placed in Coomassie Blue stain for 2.5 min and rinsed very quickly (one dip) in 70% ethanol and then 2 times in distilled water (3–4 dips in each rinse). The back of the slides was dried with tissue paper, followed by drying on a slide warmer at 37–40°C. Additional fixed and dried sperm slides were placed in Toluidine Blue stain (1% w/v in distilled water) for 15 min. The slides were rinsed 2 times in distilled water (3–4 dips in each rinse). The back of the slides was dried with tissue paper, followed by drying on a slide warmer at 37–40°C. A total of 200 sperms was analyzed morphologically under microscope for each control and treated males.

Statistical procedure

Statistical analysis was performed as described previously.[9] Briefly, means and standard deviations of means for groups, mean values of body weights, food and water consumption, absolute organ weights, relative organ weights, and hematological and biochemical parameters were calculated. Data were checked for normality using the Shapiro–Wilk and Anderson–Darling tests. Standard repeated analysis of variance was used for statistical evaluation of the data. When a significant effect was found, Tukey's multiple comparison and within-treatment contrast tests were used for further analysis. The statistical significance level was set at $P \leq 0.05$ (SYSTAT 13, Systat Inc, Chicago, IL, USA). Estimates of power were calculated using published control data for various organs and hematology in males and females and sperm velocity parameters.[14,15] The assumptions were 80% power and $\alpha = 0.05$ for a 10% and 20% two-sided treatment effect that shifted the mean but did not affect the variance.

Results

Death, abnormalities, and feeding

One female and male in the treatment group and one female in the control group died due to gavage complications, and they were excluded from histopathogical data. No other deaths or clinical abnormalities for any male or female animals were observed during the experimental period. There were no significant differences in mean body weight gains in treated females in comparison to the controls as a result of 200 mg/kg oral administration of *StemEnhance* (Figure 1). Similarly, administration of *StemEnhance* did not cause any significant changes in weekly water and food consumption in treated females in comparison to controls (Figures 2 and 3). In males, there were no significant differences in mean body weight gain and in mean weekly water and food consumption between treated and control rats (Figures 4–6).

Gross pathology

Tables 1 and 2 show the absolute and relative organ weights in treated and control females and males, respectively. There was a 12% increase in the absolute (P = 0.056) and relative (P = 0.076) mean spleen weights for treatment versus control females. Relative epididymis weights (R + L) were increased 11.1% in treated males (P = 0.045).

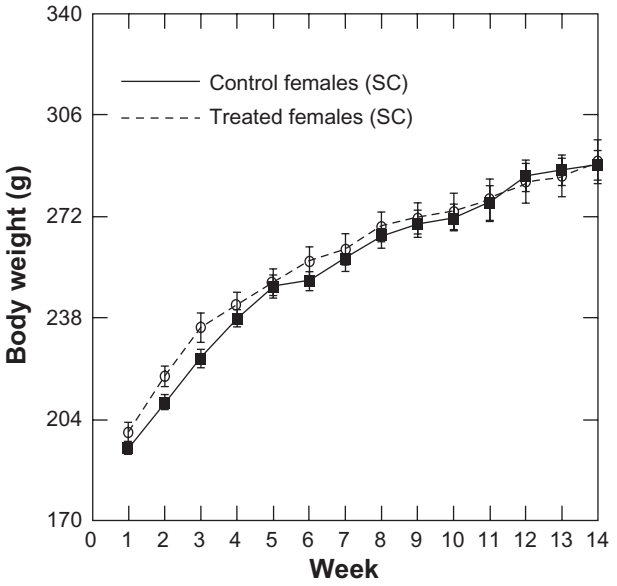

Figure 1 Mean body weight (±SD) in treated and control females (n = 12 female rats/group).

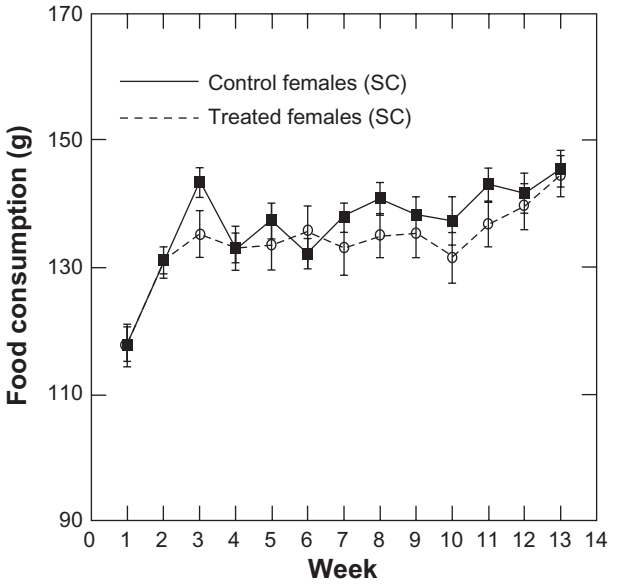

Figure 3 Mean food consumption (±SD) in treated and control females (n = 12 female rats/group).

There were no statistically significant differences in the relative or absolute weights of all other organs between treated and control groups in females or males. The increased spleen and epididymis weights were not connected with any pathological processes (per the histopathology) and therefore could not be linked with administration of *StemEnhance*.

Organ weights were also normalized to the brain.[16] Test materials that alter body weight generally do not alter brain weight, making organ-to-brain weight ratios useful in cases of notable decreases in body weight that impact organ-to-body weight ratios.[17] Organ-to-brain weight ratios also may be helpful in normalizing animal-to-animal variability.[18] There were no significant treatment differences in either males or females, for organ weights normalized by the brain weight.

Histopathology

Histopathologic lesions attributed to background lesions were identified in both the control and treated animal populations. Background lesions are pathologic lesions

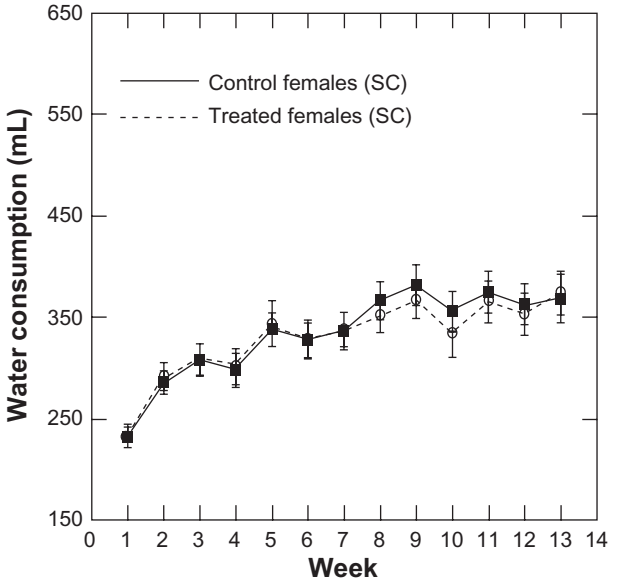

Figure 2 Mean water consumption (±SD) in treated and control females (n = 12 female rats/group).

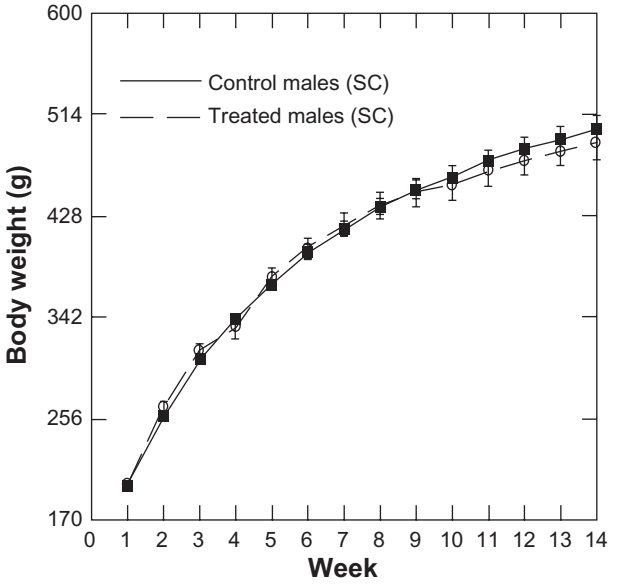

Figure 4 Mean body weights (±SD) in treated and control males (n = 12 male rats/group).

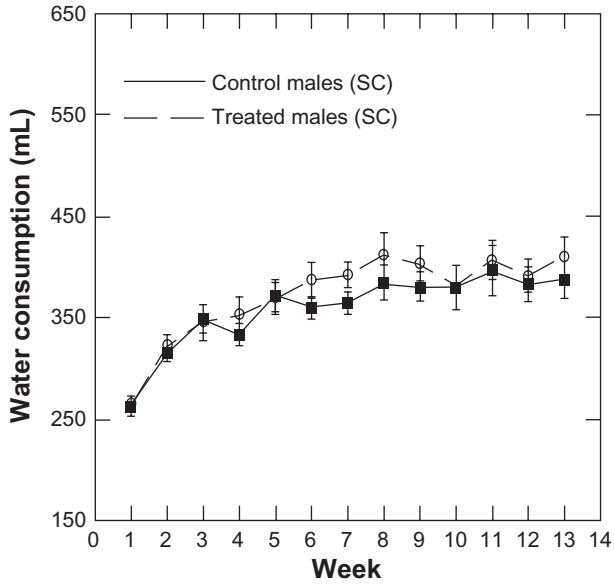

Figure 5 Mean water consumption (±SD) in treated and control males (n = 12 male rats/group).

observed in animals that are not related to specific causes including age-related lesions, spontaneous lesions (including nonspecific genetic change), or environmentally induced lesions (infectious agents, feed, bedding, cleaning agents, etc). In all cases, there were no statistically significant differences in the distribution of the numbers and types of lesions between control and treated animals within the same gender or between genders (Table 3). Thus, *StemEnhance* did not have a negative effect on any of the examined tissues.

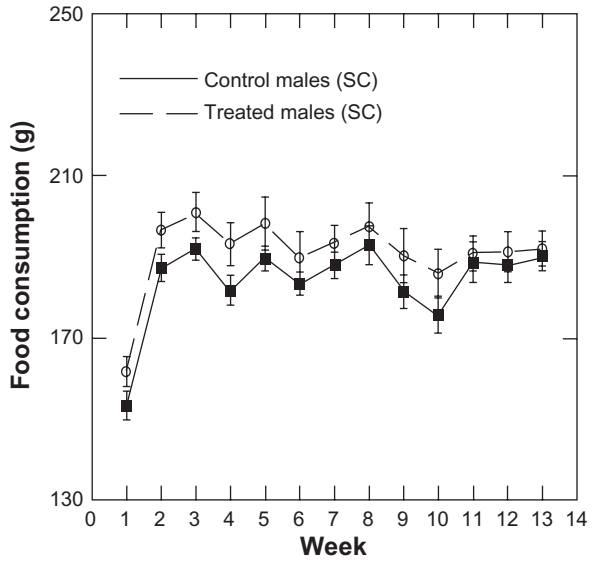

Figure 6 Mean food consumption (±SD) in treated and control males (n = 12 male rats/group).

Table 1 Summary of mean (±SD) absolute and relative organ weight values (g/100 g) in treated and control female rats (n = 12/group)

Organ	Treated	Control
Adrenals		
Absolute	0.201 ± 0.082	0.201 ± 0.078
Relative	0.071 ± 0.032	0.070 ± 0.027
Brain		
Absolute	2.05 ± 0.166	2.032 ± 0.117
Relative	0.711 ± 0.082	0.704 ± 0.053
Heart		
Absolute	0.984 ± 0.080	0.994 ± 0.106
Relative	0.340 ± 0.034	0.344 ± 0.040
Kidney (left)		
Absolute	1.152 ± 0.110	1.085 ± 0.105
Relative	0.399 ± 0.053	0.376 ± 0.036
Kidney (right)		
Absolute	1.159 ± 0.098	1.103 ± 0.099
Relative	0.402 ± 0.044	0.382 ± 0.030
Liver		
Absolute	10.69 ± 1.05	10.26 ± 0.901
Relative	3.68 ± 0.262	3.55 ± 0.223
Ovary (left)		
Absolute	0.144 ± 0.064	0.159 ± 0.062
Relative	0.050 ± 0.024	0.055 ± 0.022
Ovary (right)		
Absolute	0.137 ± 0.049	0.131 ± 0.063
Relative	0.047 ± 0.017	0.045 ± 0.021
Spleen		
Absolute	0.747 ± 0.095	0.667 ± 0.091
Relative	0.259 ± 0.039	0.231 ± 0.031
Uterus		
Absolute	0.616 ± 0.137	0.672 ± 0.140
Relative	0.214 ± 0.049	0.234 ± 0.055

Hematology

The hematological findings in females (Table 4) and males (Table 5) did not show any consistent treatment-related effects. Values for some controls and some treated animals sporadically varied from the Charles River Laboratories reference range. There were no statistically significant differences in mean Hb, RBC, PCV, MCV, MCH, and MCHC values at any time points between the treated and control females and also between the treated and control males.

Mean WBC counts in all females and all males were within the reference range throughout the study. Mean WBC counts did not differ between groups at any time.

There were sporadic changes in neutrophil, lymphocyte, monocyte, and eosinophil counts in treated and control animals. Mean neutrophil, lymphocyte, monocyte, and eosinophil cell counts were within the reference ranges indicated for neutrophil (1%–32%), lymphocytes (67%–98%),

Table 2 Summary of mean (±SD) absolute and relative organ weight values (g/100 g) in treated and control male rats (n = 12/group)

Organ	Treated	Control
Adrenals		
Absolute	0.216 ± 0.086	0.198 ± 0.081
Relative	0.044 ± 0.017	0.039 ± 0.015
Brain		
Absolute	2.246 ± 0.144	2.185 ± 0.149
Relative	0.461 ± 0.040	0.437 ± 0.040
Epididymis (left)		
Absolute	0.772 ± 0.071	0.717 ± 0.098
Relative	0.158 ± 0.016	0.143 ± 0.019
Epididymis (right)		
Absolute	0.790 ± 0.067	0.728 ± 0.089
Relative	0.162 ± 0.016	0.146 ± 0.018
Heart		
Absolute	1.505 ± 0.243	1.516 ± 0.282
Relative	0.306 ± 0.025	0.302 ± 0.049
Kidney (left)		
Absolute	1.877 ± 0.193	1.857 ± 0.162
Relative	0.383 ± 0.020	0.371 ± 0.033
Kidney (right)		
Absolute	1.913 ± 0.205	1.888 ± 0.233
Relative	0.390 ± 0.015	0.377 ± 0.042
Liver		
Absolute	19.53 ± 2.960	19.66 ± 2.150
Relative	3.972 ± 0.290	3.915 ± 0.274
Spleen		
Absolute	1.005 ± 0.133	0.986 ± 0.145
Relative	0.205 ± 0.022	0.196 ± 0.021
Testis (left)		
Absolute	1.984 ± 0.195	1.954 ± 0.159
Relative	0.406 ± 0.037	0.391 ± 0.035
Testis (right)		
Absolute	2.053 ± 0.162	1.975 ± 0.260
Relative	0.420 ± 0.030	0.395 ± 0.055

monocytes (0%–6%), and eosinophils (0%–3%) for females (Table 4) and males (Table 5) throughout the study.

Clinical biochemistry

There were no consistent significant differences between treated and control animals in the serum analyses for females (Table 6) or males (Table 7). Most means and individual concentrations in treated and control females were within the reference range throughout the study. The differences are described below.

The mean ALT concentrations in treated and control females were within the reference range (23–67 U/L) (Table 6) before the dosing started. The mean ALT concentration for treated females (54.00 U/L), but not for control females (75.91 U/L), was within the reference range at the end of the dosing. Mean ALT concentrations in treated and control males were within the reference range (24–49 U/L) (Table 7) before dosing started but not at the end of the dosing period (55.7 and 64.4 U/L, treated and control, respectively). The results of histopathological examination of the livers from animals that had high concentrations of ALT levels did not show any hepatic changes associated with subchronic hepatotoxic episode.[19]

Table 3 Summary of histologic lesions of *StemEnhance*™ in treated and control animals

	Control		Treated	
	Female	Male	Female	Male
No lesions	3/11	3/12	0/11	2/11
Nasal cavity	0/11	0/12	0/11	2/11
Esophagus	0/11	0/12	1/11	0/11
Trachea	0/11	1/12	2/11	0/11
Mesenteric lymph node	0/11	0/12	3/11	0/11
Submandibular lymph node	4/11	0/12	2/11	1/11
Tracheobronchial lymph node	1/11	0/12	1/11	0/11
Harderian gland	2/11	4/12	4/11	2/11
Liver	0/11	0/12	0/11	0/11
Stomach	0/11	0/12	2/11	2/11
Intestine	1/11	1/12	2/11	0/11
Preclitoral gland	1/11	NA	3/11	NA
Preputial gland	NA	3/12	NA	2/11
Kidney	0/11	1/12	1/11	1/11
Bone marrow	0/11	0/12	1/11	2/11
Mammary gland	0/11	NA	0/11	NA
Prostate gland	NA	5/12	NA	1/11
Urinary bladder	0/11	0/12	0/11	0/11
Parathyroid gland	0/11	0/12	0/11	1/11
Pituitary gland	1/11	0/12	0/11	0/11

Abbreviation: NA, not applicable.

The mean calcium concentrations both in treated and control animals were not within the reference range for females (8.24–10.36 mg/dL) (Table 6) or males (8.08–9.92 mg/dL) (Table 7) throughout the study. The mean phosphorus concentrations in treated and control females and males are shown in Tables 6 and 7, respectively. There were no statistically significant differences in mean phosphorus or mean calcium concentrations between the treated and control females or between the treated and control males at any time points.

The mean potassium concentrations were within the reference range both in treated and control females (4.8–7.2 mmol/L) (Table 6) and males (5.2–7.8 mmol/L) (Table 7) throughout the study. The mean chloride concentrations were within the reference range in both treated and control females in comparison with the reference range for females (99–114 mmol/L) (Table 6) before the dosing started. Although the mean chloride concentration was within the reference range in control females, the mean in treated females (98.82 mmol/L) was slightly lower than

Table 4 Mean hematology parameters (±SD) in treated and control female rats before dosing commencement and at the end of the administration period (n = 12/group)

Parameters	Before dosing		After dosing	
	Treated	Control	Treated	Control
Eosinophils	1.33 ± 0.89	1.50 ± 0.80	3.00 ± 1.48	2.73 ± 1.85
Hemoglobin (g/dL)	15.74 ± 0.58	14.98 ± 0.47	15.00 ± 0.50	15.20 ± 0.52
Lymphocytes	86.58 ± 4.21	83.67 ± 7.02	83.82 ± 5.64	83.82 ± 5.06
Mean corpuscular hemoglobin (pg)	19.31 ± 0.57	19.53 ± 0.62	18.40 ± 0.50	18.66 ± 0.64
Mean corpuscular hemoglobin Concentration (g/dL)	35.03 ± 0.38	35.44 ± 0.22	34.67 ± 0.42	34.76 ± 0.29
Mean corpuscular volume (fL)	55.13 ± 1.69	55.13 ± 1.58	53.06 ± 1.28	53.69 ± 1.84
Monocytes	3.58 ± 2.19	2.83 ± 2.17	3.09 ± 1.38	3.64 ± 2.84
Neutrophils	8.58 ± 4.14	11.92 ± 6.42	10.36 ± 3.11	9.82 ± 3.71
Packed cell volume (%)	44.97 ± 1.65	42.28 ± 1.34	43.33 ± 1.33	43.75 ± 1.58
Red blood cells (M/μL)	8.16 ± 0.38	7.68 ± 0.30	8.17 ± 0.28	8.15 ± 0.33
White blood cells (10^3/μL)	11.14 ± 3.26	10.45 ± 2.27	8.07 ± 3.30	7.06 ± 1.40

the reference range after dosing. The mean chloride concentrations were slightly lower than the reference range (102–109 mmol/L) (Table 7) in treated and control males throughout the study. There were no statistically significant differences in mean potassium or mean chloride concentrations between the treated and control females and also between the treated and control males at any time point.

The mean sodium concentrations in the treated and control females were within the reference range (139–150 mmol/L) throughout the study (Table 6). The mean sodium concentrations were within the reference range both in treated and in control males at the end of the dosing period (Table 7). There were no statistically significant differences in mean sodium concentrations between the treated and control females and also between the treated and control males at any time point. At the start of the study, chloride, potassium, and sodium were highly correlated in both groups, whereas at termination, there were no significant correlations among these constituents in either group.

The mean glucose concentrations were not within the reference range in control and treated females (Table 6) (85–132 mg/dL) and males (Table 7) (100–130 mg/dL) before and after dosing. Almost all the females and males in the treatment and control groups had glucose concentrations slightly above reference range throughout the study. There were no statistically significant differences in mean glucose concentrations between the treated and control females and also between the treated and control males at any time point.

All mean cholesterol concentrations were within the reference range for females (27.3–97.5 mg/dL) (Table 6) throughout the study and did not differ significantly at any time point.

Table 5 Mean hematology parameters (±SD) in treated and control male rats before dosing commencement and at the end of the administration period (n = 12/group)

Parameters	Before dosing		After dosing	
	Treated	Control	Treated	Control
Eosinophils	1.42 ± 1.17	1.50 ± 1.17	2.09 ± 1.30	2.42 ± 1.78
Hemoglobin (g/dL)	14.18 ± 0.86	14.51 ± 0.58	15.45 ± 0.66	15.63 ± 0.42
Lymphocytes	83.25 ± 4.37	84.08 ± 3.48	82.82 ± 4.64	81.33 ± 6.40
Mean corpuscular hemoglobin (pg)	20.89 ± 0.69	20.40 ± 0.67	17.85 ± 0.42	17.74 ± 0.50
Mean corpuscular hemoglobin concentration (g/dL)	35.00 ± 0.35	35.02 ± 0.45	34.55 ± 0.54	34.89 ± 0.39
Mean corpuscular volume (fL)	59.70 ± 1.63	58.27 ± 1.54	51.71 ± 0.90	50.79 ± 1.22
Monocytes	4.00 ± 2.05	3.75 ± 1.55	3.00 ± 2.00	3.92 ± 3.34
Neutrophils	11.42 ± 3.63	10.58 ± 3.00	12.18 ± 4.42	12.33 ± 5.14
Packed cell volume (%)	40.58 ± 2.25	41.47 ± 1.66	44.69 ± 1.78	44.84 ± 1.21
Red blood cells (M/μL)	6.80 ± 0.37	7.12 ± 0.28	8.64 ± 0.33	8.83 ± 0.33
White blood cells (10^3/μL)	12.19 ± 2.81	14.63 ± 4.26	9.12 ± 2.93	10.05 ± 2.59

Table 6 Mean serum chemistry parameters (±SD) in treated and control female rats before dosing commencement and at the end of the administration period (n = 12/group)

Parameters	Before dosing		After dosing	
	Treated	Control	Treated	Control
Albumin (g/dL)	4.65 ± 0.23	4.76 ± 0.18	4.69 ± 0.18	4.99 ± 0.25
ALP (IU)	123.92 ± 16.04	109.17 ± 13.36	73.91 ± 12.09	63.45 ± 11.17
ALT (IU)	40.83 ± 4.65	36.42 ± 6.35	54.00 ± 10.45	75.91 ± 30.87
Anion gap (H)	24.63 ± 2.21	24.86 ± 2.77	11.60 ± 2.06	12.82 ± 2.65
Bicarbonate (mmol/L)	23.40 ± 1.34	21.99 ± 2.76	42.79 ± 2.10	40.65 ± 3.78
BUN (mg/dL)	20.70 ± 2.64	19.43 ± 1.94	23.12 ± 3.55	21.87 ± 2.22
Calcium (mg/dL)	11.23 ± 0.28	11.23 ± 0.32	12.08 ± 0.38	12.15 ± 0.36
Chloride (mmol/L)	99.08 ± 1.08	101.33 ± 1.44	98.82 ± 1.83	100.27 ± 2.24
Cholesterol (mg/dL)	67.42 ± 13.45	64.83 ± 11.01	66.91 ± 9.31	70.64 ± 7.07
Creatinine (mg/dL)	0.28 ± 0.04	0.26 ± 0.05	0.24 ± 0.05	0.31 ± 0.07
GGT	0.00 ±	0.00 ±	0.00 ±	0.00 ±
Globulin (g/dL)	1.60 ± 0.12	1.50 ± 0.10	1.90 ± 0.14	1.86 ± 0.12
Glucose (mg/dL)	144.92 ± 15.47	142.92 ± 12.06	158.09 ± 18.29	180.00 ± 30.50
Phosphorus (mg/dL)	8.00 ± 0.47	7.88 ± 0.94	6.37 ± 1.11	6.13 ± 1.20
Potassium (mmol/L)	5.44 ± 0.46	5.35 ± 0.54	6.21 ± 1.84	6.02 ± 0.45
Sodium (mmol/L)	141.67 ± 1.44	142.83 ± 1.59	147.00 ± 1.73	147.73 ± 1.42
Total protein (g/dL)	6.25 ± 0.27	6.26 ± 0.20	6.75 ± 0.18	6.85 ± 0.25
Triglycerides (mg/dL)	71.50 ± 21.98	76.00 ± 31.89	133.00 ± 53.53	113.70 ± 45.23

Abbreviations: GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

Mean cholesterol concentrations at the end of the dosing period were within the reference range (42.9–78 mg/dL) in treated males but not in control males (86.75 mg/dL; Table 7). Administration of *StemEnhance* reduced the mean cholesterol concentrations in treated males and females in comparison to the predosing values, while in control males and females, the mean cholesterol concentration at the end of the dosing period was slightly above the predosing mean. Mean triglycerides concentrations in the treatment and control female groups were within the reference range (35.5–301.7 mg/dL) (Table 6) throughout the study. Although the mean triglycerides concentrations were within the reference range (35.5–186.3 mg/dL)

Table 7 Mean serum chemistry parameters (±SD) in treated and control male rats before dosing commencement and at the end of the administration period (n = 12/group)

Parameters	Before dosing		After dosing	
	Treated	Control	Treated	Control
Albumin (g/dL)	4.43 ± 0.10	4.47 ± 0.09	4.53 ± 0.10	4.61 ± 0.13
ALP (IU)	231.25 ± 34.57	199.00 ± 19.81	100.64 ± 11.46	100.58 ± 16.84
ALT (IU)	40.92 ± 5.74	36.25 ± 5.15	55.73 ± 9.79	64.42 ± 21.49
Anion gap (H)	23.33 ± 2.00	22.37 ± 1.82	12.06 ± 3.85	12.73 ± 3.05
Bicarbonate (mmol/L)	25.86 ± 1.57	26.84 ± 1.29	45.25 ± 5.66	44.70 ± 4.85
BUN (mg/dL)	20.19 ± 2.83	17.07 ± 1.58	22.67 ± 2.31	23.18 ± 1.56
Calcium (mg/dL)	11.49 ± 0.19	11.53 ± 0.26	12.09 ± 0.24	12.18 ± 0.35
Chloride (mmol/L)	97.58 ± 1.78	98.00 ± 0.95	97.73 ± 1.35	97.25 ± 1.91
Cholesterol (mg/dL)	88.75 ± 18.99	85.42 ± 8.88	77.18 ± 13.73	86.75 ± 10.10
Creatinine (mg/dL)	0.20 ± 0.00	0.20 ± 0.00	0.24 ± 0.05	0.27 ± 0.05
GGT	0.00 ±	0.25 ± 0.87	0.00 ±	0.00 ±
Globulin (g/dL)	1.58 ± 0.15	1.60 ± 0.09	2.18 ± 0.14	2.13 ± 0.14
Glucose (mg/dL)	137.83 ± 12.55	145.83 ± 17.15	161.55 ± 32.97	143.50 ± 9.39
Phosphorus (mg/dL)	9.80 ± 0.48	10.80 ± 0.32	7.20 ± 0.92	6.69 ± 0.49
Potassium (mmol/L)	6.61 ± 0.50	6.13 ± 0.38	5.85 ± 0.48	5.93 ± 0.61
Sodium (mmol/L)	140.17 ± 1.03	141.08 ± 1.16	148.45 ± 1.51	148.75 ± 1.71
Total protein (g/dL)	6.01 ± 0.22	6.07 ± 0.12	6.71 ± 0.20	6.74 ± 0.24
Triglycerides (mg/dL)	145.50 ± 45.49	143.40 ± 47.30	162.90 ± 53.71	220.30 ± 80.48

Abbreviations: GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

in treated males at the end of the dosing period, the mean for the control males (220.3 g/dL) was above the reference range (Table 7). The mean triglycerides concentration at the end of the dosing period was significantly higher in treated females (133 g/dL) than in control females (113.7 g/dL), while the mean triglycerides concentration at the end of the dosing period was significantly lower in treated males (162.9 g/dL) than in control males (220.3 g/dL).

The mean creatinine concentrations in treated and control females were below the reference ranges for female (0.42–0.60 mg/dL) (Table 6) throughout the study. Subsequently, the mean creatinine concentrations in treated and control males were below the reference ranges for male Wistar rats (0.35–0.55 mg/dL) (Table 7). All the females and males in the treated and control groups had creatinine concentrations below the reference range throughout the study. The mean BUN concentrations in control and treated females were within the reference range (17–26 mg/dL) (Table 6) throughout the study. The mean BUN concentrations in control and treated males were within the reference range for males (16–23 mg/dL) (Table 7) before dosing. After dosing, the mean BUN concentration for treated males was within the reference range, while the mean for control males (23.18 mg/dL) was at the lower boundary. There were no statistically significant differences in mean BUN concentrations at any time points between the treated and control females and also between the treated and control males.

The mean globulin concentrations in treated and control animals were below the reference ranges for female (2.4–3.9 g/dL) (Table 6) and male (2.5–3.8 g/dL) (Table 7) Wistar rats at all times. The mean albumin concentrations in treated females were within the reference range (3.7–4.9 g/dL) (Table 6) before dosing but not at the end of the dosing period. The mean albumin concentrations in treated and control males were within a reference range (3.3–4.7 g/dL) (Table 7) before dosing and at the end of the dosing period. The mean total protein concentrations in the control and treatment groups were within the reference range for females (6.3–8.6 g/dL) (Table 6) and males (6.5–7.8 g/dL) (Table 7) at the end of the dosing period, but not before the dosing started. There were no statistically significant differences in mean total protein, mean globulin, or mean albumin concentrations between the treated and control females and also between the treated and control males at any time point.

Ki-67 IHC

The monoclonal antibody Ki-67 was used to show immunohistochemical expression of proliferating cells in the uterus.[12] The antibody reacts with a nuclear protein expressed in the G1, G2, S, and M phases of the cell cycle. Examination of uterine sections stained with Ki67 did not reveal any differences in females receiving *StemEnhance* compared to control animals.

Epididymal sperm count and morphology

For the purposes of this report, we classify the sperm parameters as 'measured' and 'derived'. Table 8 gives the mean and standard deviation for measured sperm analysis parameters in treated and control males at the end of the study. The derived parameters include wobble (WOB = VAP/VCL), sperm movement index SMI = (VSL × 0.59) + (VAP × 0.37) + (LIN × 0.95) + (STR × 0.89) + (WOB × 0.83)), sperm velocity index (SVI = (VCL × 0.87) + (VSL × 0.76) + (VAP × 0.90) + (ALH × 0.92)), progressiveness ratio (PR = VSL/VCL), linearity index (LI = VSL/VAP), dance (DNC = VCL/ALH), and dance mean (DNCmean = DNC/VSL). In summary, except for elongation, there are no significant differences in the means of the treatment and control groups. A Kruskal–Wallis test of the elongation distributions was significant (P = 0.014). The variances for STR, elongation, LI, and dance are not equal. Table 9 summarizes the morphological sperm analysis in both treated and control males.

Discussion and conclusion

Initially, there are some issues associated with the use of published 'normal' values that need to be addressed so that our results are properly interpreted. 'Normal' values are affected by the supplier, food, and housing conditions. As our predosing data show, the 'normal' values also vary within a

Table 8 Summary of mean (±SD) of measured sperm analysis parameters in treated and control males (n = 12/group)

Parameters	Treated	Control
ALH (μm)	16.62 ± 1.84	15.91 ± 1.77
BCF (Hz)	22.76 ± 2.39	22.86 ± 2.83
LIN (%)	37.73 ± 2.83	38.83 ± 4.63
Motile sperm (%)	84.40 ± 5.50	67.80 ± 23.30
Progressive motility (%)	18.50 ± 3.63	14.42 ± 6.80
Sperm count (M/mL)	31.50 ± 9.36	24.82 ± 13.50
STR (%)	66.64 ± 1.91	68.00 ± 3.91
VAP (μm/sec)	205.90 ± 26.10	195.17 ± 25.85
VCL (μm/sec)	388.40 ± 42.15	368.58 ± 47.93
VSL (μm/sec)	142.40 ± 22.51	136.60 ± 22.60
Elongation	30.91 ± 1.81	27.00 ± 4.47

Abbreviations: VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

cohort of animals. Consequently, the reference ranges should only be used as a point of reference and do not replace the need for control animals in large studies designed to assess the toxic potential of a test material. There is no substitute for age- and sex-matched control animals from the same supplier undergoing the same procedures as the test animals. Reference ranges should never be relied upon as the sole means of making judgments concerning the biological importance of a test material–related effect.[20] For example, mean reference values for ALP (IU) in males from four studies in different laboratories/breeders were 130, 137, 40, and 235.[11,21] Historical control data for ALP from 80 studies by a single breeder (RCC Ltd, Harlan Laboratories, Inc) had the following variation in 19- to 40-week-old Wistar–Hannover rats: mean of means = 59, standard deviation of means = 6.1, minimum of means = 46.5, and maximum of means = 79.4. Furthermore, because reported normal values vary widely, 'values outside of the reference range do not necessarily indicate an abnormal condition and values within the reference range do not necessarily signify a normal condition'.[19] Occasionally, very small statistically significant differences occur between control and treated groups in toxicology studies, and unfortunately, the mechanism for the difference is usually not apparent.

Since the liver is the primary organ that detoxifies toxins, a liver function test is an important indicator of the safety of *StemEnhance*. Fostser and Wians provided a useful criterion of liver damage: increased serum levels of GGT in the presence of increased serum levels of ALP strongly suggest that the ALP originated from the liver.[22] Normal serum levels of GGT in the presence of increased serum levels of ALP, however, do not necessarily mean that the ALP originated from bone. At the end of the dosing period, for all animals, GGT concentrations were within the reference range, and we conclude from Wians's criterion that there is no evidence for liver damage or impaired liver function in any animal.

Another indicator of liver disease is the BUN–creatinine ratio. The ratio increases with renal disease but factors including liver function, dietary protein intake, and muscle mass can also affect results. Although BUN levels alone can increase with renal dysfunction, they also increase with dehydration and a high-protein diet. Creatinine does not increase with dehydration alone but definitely increases with renal dysfunction. For the controls, the BUN–creatinine ratios at 0 and 90 days were 81.2 ± 10.5 (61.7–97.0) and 82.3 ± 19.1 (49.0–121.5), respectively. For the animals receiving *StemEnhance*, the ratios were 87.7 ± 19.9 (55.0–121.4) and 100.5 ± 22.8 (64.0–135.0). At time 0, the variances did not differ between groups, whereas at 90 days, the variances were significantly different (control = 364.7, *StemEnhance* = 519.6), and the means differed significantly at 90 days but not at 0 day. However, most creatinine and BUN concentrations were within the reference range throughout the study, so taken together, there is no evidence of renal dysfunction.

In the subacute study,[9] there was a difference in sperm track speed (VCL), with a 10% reduction in the *StemEnhance* group relative to the controls. Extensive searching of the literature and discussions with experts failed to identify either a mechanism for this reduction or its clinical significance. In most published reports of adverse effects of chemicals on sperm motility, several motility parameters are affected in specific ways, and these patterns were not found for the treatment group rats. We speculated that the reduction in VCL was transitory and possibly related to the fact that the exposure/postexposure observation period was almost exactly equal to the time span for meiosis in the Wistar rat. In light of this prior finding, the determination of whether or not sperm motility differs in rats following subchronic exposure to *StemEnhance* takes on special significance. As discussed above, except for a small difference in elongation, there were no differences in any of the measured or derived sperm motility parameters (Table 8). Use of published reference values for sperm velocity measurements has to evaluate the equipment used, the computer processing, and instrument settings such as frame rate. Thus, for the same group of 30 sperm tracks of progressively motile human spermatozoa, the progression velocity (VSL) and VAP were approximately constant, but the VCL decreased as the sampling frequency decreased from 200 to 5 frames/sec.[23] Intra and interindividual variability have large coefficients

Table 9 Summary of sperm morphological analysis in treated and control males (n = 12/group)

	Headless	Tailless	Hooks	Coiled tail	Abnormal head
Treatment group					
Number of sperms	3	1	1	0	0
Control group					
Number of sperms	5	0	4	0	0

of variation: in a workshop study of human sperm, the mean interindividual coefficients of variation were 22.9%, 21.8%, and 17.5% for sperm concentration, motility, and vitality, respectively.[24] For these and other reasons, toxic effects that affect sperm motility are evaluated in relation to changes that occur in particular patterns that involve both measured and derived parameters.[25] We conclude that *StemEnhance* had no toxic effects on sperm development or function based on the following: 1) none of the well-established patterns of toxicity affecting sperm are present in either the subacute or subchronic studies, 2) statistically significant differences in the sperm data are small changes in means, and 3) abnormal testicular histopathology is absent in the subacute[9] and subchronic studies.

Treatment-related morphological abnormalities were not found in any organs/tissues examined. These findings confirm that high-dose subchronic exposure to *StemEnhance* does not lead to toxicity. The dose of *StemEnhance* tested in the rat was ~7 times higher than the maximum label-recommended daily dose for human consumption. Therefore, it would appear that no toxicological hazard is likely due to the use of *StemEnhance* at label doses.

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