Subacute toxicity study in Wistar rats fed with *StemEnhance*TM, an extract from *Aphanizomenon flos-aquae*

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Abstract: This study evaluated the safety (absence of toxicity) of *StemEnhance*TM, 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 600 mg/kg of *StemEnhance* prepared in 5% glycerin in water for 2 weeks by oral gavage followed by 2 weeks of observation. 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 ~20 times the maximum label-recommended daily dose did not produce adverse effects in Wistar rats after subacute 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. It is well established that cyanobacteria are an excellent source of bioactive compounds that include vitamins, proteins, and compounds with pharmaceutical activity and algae are cultured globally for use as a food supplement (*Spirulina plantensis*).^{1,2} AFA is primarily harvested from Upper Klamath Lake (UKL) 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 triglycerides concentrations in rats.³ 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.⁴ In vitro studies suggest that AFA has antimutagenic and antiviral activities.^{1,5}

AFA is a rich source of vitamins, minerals, and antioxidant pigments including C-phycoyanin and carotenoids, for example, β -carotene, flavacin, aphanin, and aphanicin in the epiphasic fraction and aphanizophyll in the hypophasic fraction.⁶⁻⁹ 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

However, AFA from UKL coexists with a toxic BGA, *Microcystis aeruginosa*, which can produce microcystins that are potent hepatotoxins and probable tumor promoters.¹⁰⁻¹² Because *M. aeruginosa* can be collected during the harvesting process, resulting in microcystin contamination of BGA products, the concentration

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of microcystins in AFA products is regulated. However, as it is difficult to remove microcystins, it is preferable to concentrate the beneficial fractions of AFA without concentrating microcystins.

*StemEnhance*TM (STEM Tech Health Sciences, Inc., Klamath Falls OR) is a blend of two fractions extracted from AFA. One extract, which contains an L-selectin ligand, supports the release of stem cells (CD34⁺ cells) from the bone marrow.¹³ The other extract, a polysaccharide-rich fraction named MigratoseTM, 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 limited. National (eg, US Food and Drug Administration [FDA]) and international organizations (eg, Organization for Economic Cooperation and Development [OECD]) require toxicological investigations, including short- 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 toxicological profile of *StemEnhance* in Wistar rats following daily administration of 600 mg/kg by oral gavage using a stomach tube.

Materials and methods

Animals, housing, and test material

The protocol combined OECD 401 (acute oral toxicity) and OECD 407 (repeated dose 28-day oral toxicity) protocols.^{14,15} Rather than administering a large quantity of *StemEnhance* only one time as indicated in 401 OECD guidelines, we decided to administer it for 14 days with a 14-day observation period to investigate the toxicity of *StemEnhance*. The maximum amount of *StemEnhance* that can be suspended in 1 mL water plus 5% glycerin is 100 mg. Water alone was not suitable to generate a suspension of *StemEnhance*, and therefore glycerin was added. In this study, animals were dosed with almost the maximum amount of suspension (0.6 mL of 100 mg/mL equivalent to 600 mg/kg) that can be given to a 100-mg lab animal without overnight fasting. Wistar male and female rats aged ~7 weeks were used in this study (Charles River Laboratories, Inc, Wilmington, MA). Forty-eight age- and weight-matched rats were assigned an identification number that accompanied all data from that animal throughout the study and randomized 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) and tap water ad libitum. Each day, *StemEnhance* was freshly suspended in drinking water containing 5% glycerin at 100 mg/mL concentration. A constant volume of this suspension (0.6 mL/100 g of body weight [BW]) was administered daily by oral gavage for 14 days. The dose was given at a similar time each day and adjusted as necessary to maintain a constant dose level in terms of animal BW. Control animals received an equal volume of water with 5% glycerin without *StemEnhance* using the same administration route. Both groups were observed for 14 days following the last oral dose administration. The health conditions of the animals were recorded. All animals were weighed twice a week. Measurements of food and water consumptions were made at least weekly.

Gross pathology

Prior to postmortem examination, all animals were weighed and thereafter sacrificed by CO₂ inhalation, with subsequent bleeding from the heart. Immediately after dissection, the harvested organs were trimmed off any adherent tissues and weighed in closed Petri dishes in order to prevent drying effects. Wet weight was determined for the following organs: liver, brain, heart, kidneys, spleen, adrenals, testes, epididymis, uterus, and ovaries. Organ weights were expressed as absolute values (g) and to BW (g/100 g BW).

Histopathology

Complete histopathology was carried out on organs and tissues as listed above and additionally, on the small and large intestines and urinary bladder of all animals. 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).

Hematology and clinical biochemistry

Blood samples were collected from the lateral tail vein of the rats before dosing commenced, at the end of the 14-day administration period, and during necropsy directly from the heart. Standard serum chemistry profiles were performed by the College of Veterinary Medicine Diagnostic Laboratory using automated technology (Hitachi 917; Roche Diagnostics, Indianapolis, IN). Automated complete blood counts were performed on the Cell Dyn 3700 (Abbott Diagnostics, Abbott Park, IL). Blood smears were manually evaluated and 100 cell differential counts performed. The following hematological examinations were made: hematocrit (Hct) index (%), hemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leukocyte count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Clinical biochemical analyses of blood included calcium, phosphorus, creatinine, total protein, albumin, globulin, glucose, cholesterol, bicarbonate, anion gap, sodium, potassium, chloride, triglycerides, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and blood urea nitrogen (BUN). For hematology and serum chemistry parameters, Charles River Laboratory data¹⁶ were used as a reference range with the exception of the following serum chemistry parameters for which Boehm et al¹⁷ data were used; ALT, AST, calcium, cholesterol, creatinine, GGT, and triglycerides.

Ki-67 immunohistochemistry

Ki-67 staining is a well-established marker of proliferative activity.¹⁸ 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 and image analysis immunohistochemistry for Ki-67. For analysis of Ki-67 expression, 5- μ m thick slides were cut from formalin-fixed, paraffin-embedded blocks and placed in a plastic coplin jar filled with 10 mM citrate buffer (pH 6.0) and heated for 1 h, after which they were allowed to cool for ~20 min and then rinsed in tap water. Next, slides were immersed in a 3% solution of hydrogen peroxide in order to inactivate endogenous peroxidase activity. Then, the slides were rinsed in tap water followed by immersion in tris-buffered saline (TBS) (pH 7.6). After excess TBS was blotted from the slides, the slides were incubated for 30 min in Rodent Block R (Biocare Medical, Concord, CA) to block rat immunoglobulins from binding to the antimouse secondary. The slides were then rinsed in two changes of TBS

for 5 min each. The blocking solution was blotted off, and the Ki-67 primary antibody (1:3000 diluted in phosphate-buffered saline) was applied. The slides were then placed in a refrigerator to incubate overnight at 4°C. The next morning, the slides were washed in TBS 3 times for 5 min each. Mouse antirat secondary antibodies (horseradish peroxidase conjugated) (Biocare Medical) was then applied for 30 min followed by three 5-min washes in TBS. Next, diaminobenzidine tetrahydrochloride (DAB) was applied, and the slides were incubated for 5 min. After the slides were rinsed with TBS, an aqueous solution of copper chloride was applied to make the DAB reaction product more black. After rinsing, the slides were counterstained with hematoxylin (Surgipath Medical Ind., Inc, Richmond, IL). The slides were dehydrated through a series of graded alcohols and then transferred to xylene. The slides were covered with a coverslip using Permount (Fisher Scientific) as the mounting medium. Uterine tissue staining positive for Ki-67 was determined by a virtual microscopic image analysis method.

Epididymal sperm count and motility

At necropsy, the right cauda epididymis was immediately removed, weighed, and the tissue was minced in 4 mL of dmKRBT (2 M NaCl, 1 M KCl, 1 M CaCl₂, 0.01 M NaHCO₃ (prepared by dissolving 0.042 g of NaHCO₃ in 50 mL of media), 1 M MgSO₄ 7H₂O, 1 M NaH₂PO₄, dextrose, sodium pyruvate, TAPSO (3-(*N*-tris(hydroxymethyl)methylamino)-2-hydroxypropanesulfonic acid), sucrose, and bovine serum albumin (BSA), pH 7.3.

The sperm suspension was incubated at 37°C for 5 min and then 100 μ L of it was again diluted with 900 μ L of dmKRBT and vortex mixed for a couple of seconds. Fifteen microliters of this diluted sperm suspension was loaded on the hemocytometer and analyzed by computer-assisted semen analysis using a Hamilton Thorne IVOS-12.1 sperm analyzer (IVOS Version 12.1; Hamilton Thorne, Beverly, MA). Sperm counts were determined in six standard viewing fields and calculated per cauda and per gram of testis. A minimum of 200 sperms were 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 (BCF), straightness (STR), and linearity (LIN). Percentage progressive sperm is the ratio of the number of motile sperm with VAP > V₀ and STR > S₀ to the total number of sperm.¹⁹

Statistical procedure

Means and standard deviations of means for groups, mean values of BWs, food and water consumptions, 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 (RMANOVA) was used for statistical evaluation of the data. When a significant effect was found, further analysis was carried out using Tukey's multiple comparison and within-treatment contrast tests. The statistical significance level was set at $P \leq 0.05$ (Systat 13; Systat, Inc, Chicago, IL). Estimates of power were calculated using published control data for various organs and hematology in males and females and sperm velocity parameters.^{20,21} 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

No deaths or clinical abnormalities for any male or female animal were observed during the experimental period. There were no significant differences in BW gains in treated females in comparison to the control group as a result of 600 mg/kg oral administration of *StemEnhance* (Figure 1). Similarly, administration of *StemEnhance* did not cause any significant changes in weekly water (Figure 2) and food consumption (Figure 3) in treated females compared with

Figure 2 Mean weekly water consumption ($\pm$ SD) in treated and control animals. **Notes:** Upper = males, lower = females, solid = control, dashed = treated, $n = 12$ male and 12 female rats/group.

control females. There were no significant differences in BW gains (Figure 1) and in weekly water (Figure 2) and food consumption (Figure 3) between treated and control males as a result of *StemEnhance* administration and were within the reference range.

Gross pathology

There were no differences in gross pathology for any organ. Tables 1 and 2 show the absolute and relative organ weights

Figure 1 Mean weekly body weight ($\pm$ SD) in treated and control animals. **Notes:** Upper = males, lower = females, solid = control, dashed = treated, $n = 12$ male and 12 female rats/group.

Figure 3 Mean weekly food consumption ($\pm$ SD) in treated and control animals. **Notes:** Upper = males, lower = females, solid = control, dashed = treated, $n = 12$ male and 12 female rats/group.

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

Organ	Treated	Control
Liver		
Absolute	10.33 $\pm$ 0.911	10.31 $\pm$ 0.979
Relative	4.15 $\pm$ 0.327	4.21 $\pm$ 0.369
Brain		
Absolute	1.93 $\pm$ 0.063	1.92 $\pm$ 0.095
Relative	0.775 $\pm$ 0.049	0.785 $\pm$ 0.045
Heart		
Absolute	0.80 $\pm$ 0.057	0.82 $\pm$ 0.066
Relative	0.321 $\pm$ 0.021	0.336 $\pm$ 0.020
Spleen		
Absolute	0.67 $\pm$ 0.070	0.63 $\pm$ 0.190
Relative	0.268 $\pm$ 0.029	0.257 $\pm$ 0.077
Left kidney		
Absolute	0.97 $\pm$ 0.071	0.96 $\pm$ 0.076
Relative	0.389 $\pm$ 0.032	0.391 $\pm$ 0.023
Right kidney		
Absolute	0.99 $\pm$ 0.057	0.99 $\pm$ 0.115
Relative	0.397 $\pm$ 0.019	0.404 $\pm$ 0.041
Left adrenal		
Absolute	0.048 $\pm$ 0.010	0.046 $\pm$ 0.0063
Relative	0.019 $\pm$ 0.0044	0.019 $\pm$ 0.0027
Right adrenal		
Absolute	0.049 $\pm$ 0.0086	0.048 $\pm$ 0.012
Relative	0.0196 $\pm$ 0.0035	0.0197 $\pm$ 0.0050
Uterus		
Absolute	0.47 $\pm$ 0.089	0.517 $\pm$ 0.197
Relative	0.188 $\pm$ 0.038	0.213 $\pm$ 0.087
Left ovary		
Absolute	0.071 $\pm$ 0.010	0.074 $\pm$ 0.015
Relative	0.029 $\pm$ 0.0051	0.030 $\pm$ 0.0059
Right ovary		
Absolute	0.072 $\pm$ 0.012	0.073 $\pm$ 0.008
Relative	0.029 $\pm$ 0.0048	0.030 $\pm$ 0.0038

in treated and control females and males, respectively. Absolute organ weights were similar for control and treated rats. There was a slight (<5%) decrease in the relative heart weights for treatment versus control females ($P = 0.086$). The difference in relative heart weights was not connected with any pathological processes.

For each sex, relations between organs were determined using RMANOVA. To remove scalar differences in organ weights, all the values for each organ were standardized to mean = 0 and SD = 1. The organs included for both sexes were liver, brain, heart, spleen, kidney_L, and adrenals_L; additional organs for females were uterus and ovary_L and for males were testis_L and epididymis_L. (Weights for the right kidney, adrenal, ovary, testis, and epididymis were negligibly different from the values for the left organ.) Separate analyses were carried out using the standardized organ

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

Organ	Treated	Control
Liver		
Absolute	20.06 $\pm$ 1.728	20.31 $\pm$ 2.426
Relative	4.815 $\pm$ 0.342	4.843 $\pm$ 0.364
Brain		
Absolute	2.10 $\pm$ 0.066	2.098 $\pm$ 0.104
Relative	0.505 $\pm$ 0.018	0.504 $\pm$ 0.051
Heart		
Absolute	1.361 $\pm$ 0.179	1.348 $\pm$ 0.202
Relative	0.327 $\pm$ 0.045	0.321 $\pm$ 0.032
Spleen		
Absolute	1.052 $\pm$ 0.186	0.989 $\pm$ 0.183
Relative	0.252 $\pm$ 0.041	0.235 $\pm$ 0.036
Left kidney		
Absolute	1.723 $\pm$ 0.096	1.717 $\pm$ 0.156
Relative	0.414 $\pm$ 0.020	0.410 $\pm$ 0.018
Right kidney		
Absolute	1.753 $\pm$ 0.153	1.752 $\pm$ 0.214
Relative	0.421 $\pm$ 0.028	0.418 $\pm$ 0.036
Left adrenal		
Absolute	0.0427 $\pm$ 0.0125	0.0421 $\pm$ 0.0085
Relative	0.0103 $\pm$ 0.0031	0.0101 $\pm$ 0.0022
Right adrenal		
Absolute	0.0448 $\pm$ 0.0107	0.0414 $\pm$ 0.0083
Relative	0.0108 $\pm$ 0.0026	0.0100 $\pm$ 0.0022
Left testis		
Absolute	1.930 $\pm$ 0.168	1.773 $\pm$ 0.474
Relative	0.464 $\pm$ 0.037	0.423 $\pm$ 0.106
Right testis		
Absolute	1.910 $\pm$ 0.133	1.795 $\pm$ 0.510
Relative	0.459 $\pm$ 0.029	0.428 $\pm$ 0.115
Left epididymis		
Absolute	0.537 $\pm$ 0.0367	0.523 $\pm$ 0.098
Relative	0.129 $\pm$ 0.0076	0.125 $\pm$ 0.0242
Right epididymis		
Absolute	0.525 $\pm$ 0.0443	0.541 $\pm$ 0.069
Relative	0.126 $\pm$ 0.0084	0.130 $\pm$ 0.0174

wet weights and standardized organ weights proportional to BW. For both wet and proportional organ weights for each sex, there was no significant difference for group (all organs combined) or the organ profiles (organ $\times$ group).

Histopathology

Histopathologic lesions attributed to environmental challenges were identified in both the control and treated animal populations. Pathologic lesions involving the reproductive tract were limited to mild inflammation of the uterus, vagina, and preclitoral gland in the females and the ductus deferens gland, prostate gland, bulbourethral gland, and preputial gland in the males. In both sexes, there was a mild to moderate increase in the eosinophils populating the lamina propria

throughout the gastrointestinal tract. In the kidney, four out of 12 females from the treated group had small amounts of intratubular (distal convoluted tubules and collecting ducts) mineral deposition. Of the four females, two had pyelitis-urethritis-cystitis. Additionally, two females from the control group had pyelitis-urethritis-cystitis with no mineral deposition. Small amounts of mineral can be observed in the distal convoluted tubules and collecting ducts of normocalcemic rodents and are often associated with high levels of dietary calcium and decreased water consumption.²² In the absence of renal disease, as in this study, the mineral deposition is not considered to be clinically significant. In all cases, there were no statistically significant relations among animals with/without lesions between control and treated animals within the same gender or between genders.

Hematology

The hematological findings in females (Table 3) and males (Table 4) 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. For females, and separately for males, for all data combined irrespective of time and treatment, all pairwise correlations between Hb, RBC, and packed cell volume (PCV) were significant. 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.

The WBC counts were above the reference range in most control and treated females (Table 3) ($1.13\text{--}7.49 \times 10^3/\mu\text{L}$) and males (Table 4) ($1.96\text{--}8.25 \times 10^3/\mu\text{L}$) at one or more

time points. There were no statistically significant differences in WBC counts at any time points between the treated and control females. At termination, the mean WBC for the control males was statistically smaller (6.88; low end of reference range) than for the treated males (10.07; mid-reference range) ($P = 0.028$). The sporadic changes in WBC counts were observed both in control and treated males throughout the study. Sporadic changes in neutrophil, lymphocyte, monocyte, and eosinophil counts also occurred in both treated and control females and males. There were no statistically significant differences in any of these parameters at any time points.

Clinical biochemistry

There were no consistent significant differences between treated and control animals in the results of serum analyses for females (Table 5) or males (Table 6). The mean glucose concentrations were within the reference range both in treated and control females (76–175 mg/dL) (Table 5) and in treated and control males (70–208 mg/dL) (Table 6) throughout the study.

The mean sodium concentrations were within the reference range both in treated and control females (140–150 mmol/L) (Table 5) and in treated and control males (142–151 mmol/L) (Table 6) throughout the study. The mean potassium concentrations were higher in both treated and control animals in comparison to the reference range for females (3.31–4.9 mmol/L) (Table 5) and males (3.82–5.55 mmol/L) (Table 6) throughout the study. Similarly, the mean chloride concentrations were lower in both treated (Table 5) and control animals (Table 6) in comparison to the reference range for females (100–107 mmol/L) and males

Table 3 Mean hematology parameters ($\pm$ SD) in treated and control female rats before dosing and at the end of the administration and observation periods ($n = 12/\text{group}$)

Parameters	Before dosing		After dosing		End of observation	
	Treated	Control	Treated	Control	Treated	Control
Hb (g/dL)	15.28 $\pm$ 0.68	15.59 $\pm$ 0.81	16.57 $\pm$ 0.56	16.49 $\pm$ 0.36	15.59 $\pm$ 0.59	15.50 $\pm$ 0.34
RBC ($\text{M}/\mu\text{L}$)	7.42 $\pm$ 0.263	7.56 $\pm$ 0.523	8.20 $\pm$ 0.278	8.12 $\pm$ 0.35	8.14 $\pm$ 0.32	7.97 $\pm$ 0.42
WBC ($10^3/\mu\text{L}$)	14.85 $\pm$ 3.10	14.40 $\pm$ 2.77	12.40 $\pm$ 4.24	10.52 $\pm$ 3.64	8.41 $\pm$ 3.79	8.25 $\pm$ 2.45
PCV (%)	41.54 $\pm$ 1.78	42.70 $\pm$ 2.85	45.84 $\pm$ 1.11	46.18 $\pm$ 1.23	45.50 $\pm$ 1.43	45.15 $\pm$ 1.52
MCV (fL)	56.00 $\pm$ 1.62	56.54 $\pm$ 1.68	55.94 $\pm$ 1.46	56.96 $\pm$ 1.57	55.90 $\pm$ 0.76	56.71 $\pm$ 1.66
MCH (pg)	20.62 $\pm$ 0.45	20.65 $\pm$ 0.58	20.18 $\pm$ 0.49	20.36 $\pm$ 0.71	19.15 $\pm$ 0.49	19.50 $\pm$ 0.88
MCHC (g/dL)	36.78 $\pm$ 0.887	36.55 $\pm$ 1.414	36.09 $\pm$ 1.311	35.73 $\pm$ 0.535	34.24 $\pm$ 0.837	34.36 $\pm$ 0.824
Neutrophils	9.58 $\pm$ 3.204	9.50 $\pm$ 3.375	10.75 $\pm$ 9.545	10.83 $\pm$ 5.766	6.36 $\pm$ 4.319	7.25 $\pm$ 3.571
Lymphocytes	86.92 $\pm$ 4.188	85.90 $\pm$ 3.573	85.75 $\pm$ 12.114	84.50 $\pm$ 6.260	88.18 $\pm$ 5.896	87.58 $\pm$ 3.059
Monocytes	2.67 $\pm$ 2.015	3.30 $\pm$ 1.418	2.25 $\pm$ 1.913	3.417 $\pm$ 2.065	3.73 $\pm$ 2.412	3.58 $\pm$ 2.679
Eosinophils	0.833 $\pm$ 0.718	1.30 $\pm$ 1.160	1.417 $\pm$ 1.782	1.25 $\pm$ 1.138	1.727 $\pm$ 1.104	1.00 $\pm$ 0.853

Abbreviations: Hb, hemoglobin; RBC, erythrocyte count; WBC, differential leukocyte count; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Table 4 Mean hematology parameters ($\pm$ SD) in treated and control male rats before dosing, at the end of the administration and observation periods (n = 12/group)

Parameters	Before dosing		After dosing		End of observation	
	Treated	Control	Treated	Control	Treated	Control
Hb (g/dL)	15.30 $\pm$ 0.613	15.09 $\pm$ 0.642	16.55 $\pm$ 0.608	16.18 $\pm$ 0.612	15.09 $\pm$ 0.640	14.79 $\pm$ 0.438
RBC (M/ μ L)	7.42 $\pm$ 0.327	7.25 $\pm$ 0.302	8.18 $\pm$ 0.334	7.97 $\pm$ 0.324	7.89 $\pm$ 0.328	7.77 $\pm$ 0.304
WBC (10 ³ / μ L)	15.09 $\pm$ 3.131	15.49 $\pm$ 4.989	14.94 $\pm$ 1.690	13.68 $\pm$ 2.219	10.07 $\pm$ 3.363	6.88 $\pm$ 3.292
PCV (%)	43.10 $\pm$ 1.350	42.67 $\pm$ 1.249	46.11 $\pm$ 1.589	45.08 $\pm$ 1.671	44.09 $\pm$ 1.675	43.51 $\pm$ 1.046
MCV (fL)	58.11 $\pm$ 2.403	58.92 $\pm$ 1.973	56.43 $\pm$ 0.934	56.59 $\pm$ 1.024	55.93 $\pm$ 1.045	56.09 $\pm$ 1.286
MCH (pg)	20.62 $\pm$ 0.408	20.83 $\pm$ 0.855	20.24 $\pm$ 0.368	20.31 $\pm$ 0.412	19.13 $\pm$ 0.479	19.08 $\pm$ 0.641
MCHC (g/dL)	35.50 $\pm$ 1.167	35.38 $\pm$ 1.055	35.87 $\pm$ 0.250	35.88 $\pm$ 0.304	34.21 $\pm$ 0.566	34.00 $\pm$ 0.697
Neutrophils	10.50 $\pm$ 2.759	14.17 $\pm$ 5.458	11.25 $\pm$ 4.535	11.08 $\pm$ 4.188	10.17 $\pm$ 3.786	11.25 $\pm$ 7.111
Lymphocytes	81.90 $\pm$ 4.932	79.58 $\pm$ 6.612	81.75 $\pm$ 5.207	82.00 $\pm$ 4.411	86.00 $\pm$ 4.112	85.33 $\pm$ 8.338
Monocytes	6.30 $\pm$ 2.669	5.25 $\pm$ 3.223	5.417 $\pm$ 2.234	6.08 $\pm$ 1.881	3.33 $\pm$ 2.348	2.50 $\pm$ 1.624
Eosinophils	1.20 $\pm$ 1.229	1.00 $\pm$ 1.128	1.58 $\pm$ 1.240	0.83 $\pm$ 0.718	0.67 $\pm$ 0.779	0.92 $\pm$ 0.900

Abbreviations: Hb, hemoglobin; RBC, erythrocyte count; WBC, differential leukocyte count; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

(100–106 mmol/L) throughout the study. On the other hand, there were no statistically significant differences in potassium or chloride concentrations between the treated and control animals at any time points.

The mean calcium concentrations in both treated and control animals before dosing and at the end of the dosing period were within the reference range for females (9.7–11.2 mg/dL) (Table 5) and males (9.5–11.5 mg/dL) (Table 6). On the other hand, the mean calcium concentrations were not within the reference range in treated (12.19 mg/dL in females and 12.32 mg/dL in males) and

control animals (12.02 mg/dL in females and 12.42 mg/dL in males) at the end of the observation period. The mean phosphorus concentrations both in treated and control animals at the end of the dosing and observation periods were within the reference range for females (5.02–10.7 mg/dL) (Table 5) and males (5.58–10.41 mg/dL) (Table 6). Additionally, there were no statistically significant differences in phosphorus and calcium concentrations between the treated and control animals at any time points.

The majority of the animals in the treated and control groups had creatinine concentrations <0.2 mg/dL before the

Table 5 Mean serum chemistry parameters ($\pm$ SD) in treated and control females before and after dosing and at the end of the observation periods (n = 12/group)

Parameters	Before dosing		After dosing		End of observation	
	Treated	Control	Treated	Control	Treated	Control
Glucose (mg/dL)	122.8 $\pm$ 19.31	118.5 $\pm$ 11.54	122.4 $\pm$ 16.26	121.2 $\pm$ 9.51	166.7 $\pm$ 36.35	156.3 $\pm$ 26.84
Anion gap (H)	29.23 $\pm$ 2.235	27.75 $\pm$ 2.660	24.68 $\pm$ 2.108	26.40 $\pm$ 2.151	14.21 $\pm$ 5.221	14.96 $\pm$ 3.500
Sodium (mmol/L)	141.4 $\pm$ 1.379	140.8 $\pm$ 1.528	142.0 $\pm$ 0.739	142.2 $\pm$ 0.835	147.0 $\pm$ 1.414	146.3 $\pm$ 2.015
Potassium (mmol/L)	6.13 $\pm$ 0.458	6.23 $\pm$ 0.443	5.88 $\pm$ 0.362	5.58 $\pm$ 0.383	6.02 $\pm$ 0.607	6.33 $\pm$ 0.827
Chloride (mmol/L)	98.42 $\pm$ 1.379	97.50 $\pm$ 1.624	99.92 $\pm$ 1.084	99.25 $\pm$ 1.288	97.33 $\pm$ 2.060	97.25 $\pm$ 1.288
GGT	0	0	0.583 $\pm$ 1.379	0	0	0
ALP (IU)	216.7 $\pm$ 44.61	198.5 $\pm$ 33.56	138.4 $\pm$ 18.80	132.8 $\pm$ 25.21	107.1 $\pm$ 15.65	108.8 $\pm$ 24.66
ALT (IU)	38.58 $\pm$ 6.388	41.08 $\pm$ 8.888	72.33 $\pm$ 111.47	41.92 $\pm$ 6.557	47.83 $\pm$ 9.083	51.58 $\pm$ 12.767
BUN (mg/dL)	19.66 $\pm$ 3.341	20.38 $\pm$ 2.719	20.09 $\pm$ 1.663	21.58 $\pm$ 2.634	21.58 $\pm$ 2.929	22.32 $\pm$ 1.949
Calcium (mg/dL)	10.74 $\pm$ 0.193	10.70 $\pm$ 0.322	10.56 $\pm$ 0.239	10.38 $\pm$ 0.229	12.19 $\pm$ 0.419	12.02 $\pm$ 0.665
Phosphorus (mg/dL)	9.09 $\pm$ 0.739	8.94 $\pm$ 0.636	6.97 $\pm$ 0.438	6.99 $\pm$ 0.566	7.98 $\pm$ 0.694	7.74 $\pm$ 0.720
Creatinine (mg/dL)	<0.2 $\pm$ 0.0	<0.2 $\pm$ 0.0	0.225 $\pm$ 0.045	0.242 $\pm$ 0.052	0.308 $\pm$ 0.029	0.283 $\pm$ 0.039
Total protein (g/dL)	6.27 $\pm$ 0.210	6.28 $\pm$ 0.233	6.42 $\pm$ 0.195	6.68 $\pm$ 0.242	6.90 $\pm$ 0.186	6.91 $\pm$ 0.332
Albumin (g/dL)	4.58 $\pm$ 0.147	4.58 $\pm$ 0.175	4.66 $\pm$ 0.156	4.74 $\pm$ 0.207	5.03 $\pm$ 0.130	4.97 $\pm$ 0.227
Triglycerides (mg/dL)	86.4 $\pm$ 31.45	99.8 $\pm$ 35.85	–	–	155.7 $\pm$ 46.99	127.8 $\pm$ 42.17
Cholesterol (mg/dL)	75.75 $\pm$ 10.26	74.75 $\pm$ 12.99	72.08 $\pm$ 12.67	75.75 $\pm$ 14.74	70.08 $\pm$ 10.53	72.33 $\pm$ 12.34
Bicarbonate (mmol/L)	19.89 $\pm$ 1.436	21.81 $\pm$ 2.524	23.28 $\pm$ 2.334	22.10 $\pm$ 2.120	40.48 $\pm$ 5.579	40.46 $\pm$ 5.942
Globulin (g/dL)	1.68 $\pm$ 0.140	1.70 $\pm$ 0.154	1.76 $\pm$ 0.144	1.93 $\pm$ 0.137	1.87 $\pm$ 0.161	1.94 $\pm$ 0.173

Abbreviations: GCT, gamma glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

Table 6 Mean serum chemistry parameters ($\pm$ SD) in treated and control males before and after dosing and at the end of the observation periods (n = 12/group)

Parameters	Before dosing		After dosing		End of observation	
	Treated	Control	Treated	Control	Treated	Control
Glucose (mg/dL)	126.7 $\pm$ 11.67	133.9 $\pm$ 15.34	123.9 $\pm$ 8.73	132.9 $\pm$ 11.51	146.9 $\pm$ 14.53	148.8 $\pm$ 20.53
Anion gap (H)	27.16 $\pm$ 2.244	29.75 $\pm$ 3.310	24.18 $\pm$ 2.724	25.80 $\pm$ 1.768	15.98 $\pm$ 2.92	15.08 $\pm$ 2.405
Sodium (mmol/L)	141.8 $\pm$ 0.866	141.0 $\pm$ 1.183	144.1 $\pm$ 1.165	144.3 $\pm$ 1.485	148.4 $\pm$ 1.443	148.3 $\pm$ 1.815
Potassium (mmol/L)	6.41 $\pm$ 0.306	6.56 $\pm$ 0.832	6.36 $\pm$ 0.557	6.08 $\pm$ 0.314	6.2 $\pm$ 0.667	6.05 $\pm$ 0.450
Chloride (mmol/L)	97.42 $\pm$ 1.443	96.91 $\pm$ 2.386	98.42 $\pm$ 1.443	97.92 $\pm$ 1.311	96.25 $\pm$ 1.215	95.83 $\pm$ 1.642
GGT	0.50 $\pm$ 1.732	0	0	0	0	0
ALP (IU)	299.3 $\pm$ 46.72	279.3 $\pm$ 45.57	195.7 $\pm$ 25.74	189.9 $\pm$ 35.27	148.8 $\pm$ 22.21	150.3 $\pm$ 31.44
ALT (IU)	38.08 $\pm$ 3.87	36.73 $\pm$ 6.96	41.25 $\pm$ 5.23	38.67 $\pm$ 3.96	39.58 $\pm$ 6.07	44.25 $\pm$ 10.74
BUN (mg/dL)	18.65 $\pm$ 1.800	18.20 $\pm$ 1.860	19.42 $\pm$ 1.309	20.37 $\pm$ 1.227	22.15 $\pm$ 1.696	22.69 $\pm$ 1.237
Calcium (mg/dL)	10.84 $\pm$ 0.353	10.59 $\pm$ 0.373	11.08 $\pm$ 0.270	11.03 $\pm$ 0.325	12.32 $\pm$ 0.484	12.42 $\pm$ 0.424
Phosphorus (mg/dL)	10.39 $\pm$ 0.368	10.83 $\pm$ 0.858	8.88 $\pm$ 0.450	8.73 $\pm$ 0.519	9.08 $\pm$ 0.635	8.84 $\pm$ 0.502
Creatinine (mg/dL)	<0.2 $\pm$ 0.0	<0.2 $\pm$ 0.0	0.200 $\pm$ 0.00	0.217 $\pm$ 0.039	0.275 $\pm$ 0.045	0.258 $\pm$ 0.051
Total protein (g/dL)	6.09 $\pm$ 0.198	6.07 $\pm$ 0.241	6.69 $\pm$ 0.202	6.70 $\pm$ 0.256	6.65 $\pm$ 0.306	6.76 $\pm$ 0.329
Albumin (g/dL)	4.33 $\pm$ 0.107	4.26 $\pm$ 0.136	4.53 $\pm$ 0.137	4.47 $\pm$ 0.183	4.59 $\pm$ 0.188	4.59 $\pm$ 0.198
Triglycerides (mg/dL)	146.8 $\pm$ 49.60	150.8 $\pm$ 55.10	–	–	154.3 $\pm$ 39.23	253.2 $\pm$ 78.12
Cholesterol (mg/dL)	90.17 $\pm$ 10.03	79.91 $\pm$ 11.96	91.50 $\pm$ 11.63	87.83 $\pm$ 13.91	89.75 $\pm$ 10.87	85.50 $\pm$ 13.29
Bicarbonate (mmol/L)	23.58 $\pm$ 1.557	20.91 $\pm$ 3.867	27.85 $\pm$ 1.542	26.61 $\pm$ 1.712	42.43 $\pm$ 3.122	43.39 $\pm$ 4.172
Globulin (g/dL)	1.76 $\pm$ 0.124	1.81 $\pm$ 0.158	2.16 $\pm$ 0.144	2.23 $\pm$ 0.150	2.06 $\pm$ 0.156	2.17 $\pm$ 0.167

Abbreviations: GCT, gamma glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

experiment started, and as these were below the quantification limit, they are not reported. Subsequently, the mean creatinine concentrations in treated and control animals were within the reference ranges for female (0.2–0.6 mg/dL) (Table 5) and male (0.2–0.5 mg/dL) (Table 6) Wistar rats at the end of the dosing and observation periods.

The mean total protein, albumin, and globulin concentrations in control and treated animals were within the reference range for female (5.5–7.7 g/dL for total protein; 3.6–5.5 g/dL for albumin; 1.5–2.4 g/dL for globulin) (Table 5) and male (5.2–7.1 g/dL for total protein; 3.4–4.8 g/dL for albumin; 1.5–2.4 g/dL for globulin) (Table 6) Wistar rats at the end of the dosing and observation periods. There were no statistically significant differences in total protein, albumin, and globulin concentrations between the treated and control animals at any time points.

The change in mean triglycerides between the initial and termination concentrations differed between the control and *StemEnhance* treatments. For all groups, triglycerides were increased at termination relative to pretreatment concentrations. Triglyceride concentrations at the end of the dosing were not determined due to miscommunication with the laboratory. For females, the increases were 24.5% (*c*) and 73.9% (*t*). For males, the increases were 82.1% (*c*) and 13.9% (*t*). However, a Kolmogorov–Smirnov nonparametric test of the difference between two sample cumulative

distribution functions for females, and separately for males, was not significant at either the start or end of the study for females. There was no significant difference in distributions for treated and control males at the start ($P > 0.4$), but the difference was significant at the end of the study ($P = 0.001$).

Approximately half of the treated and control females had cholesterol concentrations slightly higher than the reference range (24–73 mg/dL) (Table 5) throughout the study. Additionally, the majority of the treated and control males had cholesterol concentrations slightly higher than the reference range (37–85 mg/dL) (Table 6) throughout the study. There were no statistically significant differences in cholesterol concentrations at any time points between the treated and control animals.

The mean ALP concentrations were within the reference ranges in treated and control females (26–147 U/L) (Table 5) and also in treated and control males (62–230 U/L) (Table 6) at the end of dosing and observation periods. There were no statistically significant differences in ALP concentrations at any time points between the treated and control animals. The mean ALT concentrations in treated and control females were within the reference range (18–45 U/L) (Table 5) before the experiment started. Two treated (48 and 426 U/L) and two control females (54 and 55 U/L) had serum ALT concentrations higher than the reference range at the end of

the dosing period. The treated female rat that had an ALT concentration of 426 U/L at the end of the dosing period had an ALT concentration of 47 U/L at the end of the observation period. The histopathological evaluations of the livers of these females indicated no lesions or histopathological changes. The mean ALT concentrations in treated and control males were within the reference range (16–48 U/L) (Table 6) throughout the study.

The mean BUN concentrations in control and treated animals were within the reference range for females (16–27 mg/dL) (Table 5) and males (15–23 mg/dL) (Table 6) throughout the study. There were no statistically significant differences in BUN concentrations at any time points between the treated and control animals.

Ki-67 immunohistochemistry

The monoclonal antibody Ki-67 was used to show immunohistochemical expression of proliferating cells in the uterus. 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 Ki-67 did not reveal any differences in females receiving *StemEnhance* compared to control animals.

Epididymal sperm count

Sperm analysis parameters in treated and control males at the end of the study are summarized in Table 7. There was a slight (~10%), statistically significant, decrease in the VCL distribution and mean in treated males versus the distribution and mean in control males. There were no other statistically significant differences in the sperm analysis parameters between treated and control groups.

Table 7 Summary of mean ($\pm$ SD) sperm analysis parameters in treated and control males (n = 12/group)

Parameters	Treated	Control
Sperm count (M/mL)	15.4 $\pm$ 7.57	15.3 $\pm$ 7.35
Motile sperm (%)	56.2 $\pm$ 25.7	57.3 $\pm$ 18.3
Progressive motility (%)	10.75 $\pm$ 4.0	10.92 $\pm$ 3.8
VAP (μ L/ms)	169.8 $\pm$ 24.2	172.8 $\pm$ 22.8
VSL (μ L/ms)	122.5 $\pm$ 34.4	123.2 $\pm$ 21.7
VCL (μ L/ms)	299 $\pm$ 28.4	335 $\pm$ 45.6
ALH (μ L)	13.3 $\pm$ 3.54	16.2 $\pm$ 3.46
BCF (Hz)	21.5 $\pm$ 3.06	22.5 $\pm$ 8.00
STR (%)	70.8 $\pm$ 8.6	71.8 $\pm$ 6.1
LIN (%)	44.3 $\pm$ 14.9	40.7 $\pm$ 4.2

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.

Discussion and conclusion

Subacute toxicity data have been used for several decades to predict the hazard of long-term, low-dose exposure to a particular compound.^{23–27} Our studies demonstrated that feeding *StemEnhance* at 600 mg/kg for 2 weeks was not toxic to Wistar rats. *StemEnhance* did not affect the behavior or physical examinations of the rats. There were no differences in weight gain or final weight between animals dosed with placebo and *StemEnhance*. *StemEnhance* did not affect hematology or clinical chemistry. Sporadic differences in some analytes were not treatment related and did not affect either gross or microscopic pathology and therefore were interpreted as biological variability normally observed in Wistar rats.^{17,20}

Treatment-related morphological abnormalities were not found in any organs/tissues examined. These findings confirm other studies showing that, as for AFA, high exposure to *StemEnhance* does not lead to toxicity.^{10,13}

The only significant difference in sperm motility due to *StemEnhance* was a 10% reduction in VCL. Examination of the literature showed that when chemicals affected sperm motility, other velocity metrics (eg, linear velocity) were also affected. We were unable to find any published study in which only VCL was retarded, and experts (personal communication with Dr Rex Hess and Dr Paul Cooke, University of Illinois at Urbana-Champaign) could not identify a known cause. Toman et al remark: ‘Decrease in VCL, even insignificant, could suggest some impairment of the motion ability as VCL is the total distance in an observation period that the sperm head can traverse’.²⁸ However, a fertilization study in rats found that ‘changes in VCL of spermatozoa were not correlated with fertilization rate’.²⁹ Furthermore, it is well documented in numerous species that sperm from different males, as well as from a single male, comprises subpopulations that differ in motility.³⁰ The cycle of the seminiferous epithelium lasts 12.8–13.3 days.^{31,32} Huckins calculated that type A stem spermatogonia evolved into leptotene primary spermatocytes in 13.3 days; leptotene cells completed meiosis and produced spermatids in 17.2 days; and spermatids matured into spermatozoa in 22.7 days.³² Treatment with *StemEnhance* took place during one cycle of the seminiferous epithelium and close to one cycle of the completion of meiosis. Similarly, the duration of the postexposure period was also the duration of meiosis. The total time of the study encompassed all the processes involved in the production of mature sperm. The differences between the

motility parameters for sperm from control and treated rats suggest that *StemEnhance* had some effect on sperm development resulting in different subpopulations in these two groups of 'normal' Wistar rats. *StemEnhance* may have affected sperm development during the cycle of the seminiferous epithelium, resulting in different subpopulations of hyperactivated sperm in these two groups of normal Wistar rats.

The dose of *StemEnhance* tested in the rat was ~20 times higher than the maximum label-recommended daily dose for human consumption. Therefore, it would appear that no toxicological hazard is related to the use of *StemEnhance* at label doses.

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Disclosure

The authors report no conflicts of interest in this work.

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