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Safety evaluation of INFAT® PLUS: Acute, genetic, teratogenic, and subchronic (90-day) toxicity studies

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ABSTRACT

INFAT®PLUS, is a sn-2 palmitate enriched fat ingredient intended for infant formula. A battery of toxicological studies was conducted in accordance with the Food Safety Toxicological Assessment GB-15193 (China), to confirm the safety of INFAT®PLUS. In the acute oral toxicity test, the LD50 of INFAT® PLUS was higher than 53.4 g /kg BW and 26.7 g/kg BW for ICR mice and SD rats, respectively. In a subchronic study, INFAT® PLUS was administered by oral gavage to SD rats with maximal daily dose of 8.90 g/kg BW for 90 days. No treatment-related clinical signs or mortalities were observed. The no-observed-adverse-effect level (NOAEL) was set at 8.90 g/kg BW. Similarly, no evidence of genotoxicity effect was noted in several in vitro and in vivo tests, including bacterial reverse mutation (Ames) test, mouse erythrocyte micronucleus test, and chromosome aberration test of mouse spermatogonia/spermatocyte. For the teratogenic evaluations, no toxicological signs were observed in both pregnant SD rat and fetuses, and the NOAEL of INFAT® PLUS was determined to be 8.90 g/kg BW. Based on the obtained results we concluded that INFAT® PLUS was found non-toxic under the experimental conditions, and the totality of the safety data supports its use for infant nutrition.

1. Introduction

Human milk (HM) is considered the best source of nutrition for infants, and it is widely believed to be tailor made, meeting the precise and unique needs of each mother-infant pair [1,2]. Besides its nutritional functions, HM promotes both short- and long-term health benefits including modifying the function of the gastrointestinal tract and the immune system as well as reduced risk of obesity and type 2 diabetes [3,4]. Among the most important components of HM are fats, providing almost 50 % of the energy intake required for the development and growth of newborns [5]. As part, triacylglycerols (TAGs) are the major form of fat, representing about 98 % of total lipids [6]. The properties of TAGs, and subsequently their nutritional and physiological function, are largely affected by their Fatty acids (FAs) composition and distribution along the glycerol backbone [7]. The main FAs in HM lipids are palmitic (16:0), oleic (18:1n-9) and linoleic (18:2n-6) acids, accounting for more than 70 % of all FAs. Palmitic acid is mainly located at the *sn-2* position (*sn-2* palmitate), while unsaturated fatty acids are found at the sn-1,3 (outer) positions [6,8].

Compared with the other macronutrients, fatty acids are one of the most variable components of human milk, largely affected by maternal diet pattern, especially across various geographic regions which have different diet characteristics [8–10]. In this regard, it has been suggested that the C52 TAGs composition of Chinese breast milk is substantially different from breast milk in other countries. Data collected from human milk studies showed that the main C52 TAG of western HM is Oleic-Palmitic-Oleic (OPO, 18:1n-9–16:0–18:1n-9) whereas in Chinese HM the C52 TAG of Oleic-Palmitic-Linoleic (OPL, 18:1n-9–16:0–18:2n-6) is found to be more predominant [11–15]. The source of this difference originates from higher levels of linoleic acid

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Table 1
Composition of INFAT® PLUS (Batch no. 0002013196).

Component	Value
C52 triglycerides, w/w (%)	42.8
OPL/OPO ratio, w/w (%)	0.94
Out of total fatty acids, w/w (%)	
Palmitic acid (C16:0)	37.9
Stearic acid (C18:0)	5.8
Oleic acid (C18:1 n-9)	35.5
Linoleic acid (C18:2 n-6)	18.0
Sum of other fatty acids	2.8
Palmitic acid (C16:0) in sn-2 position[a] (%)	53.6
Peroxide value (meq/kg)	<0.1
Free fatty acid as oleic acid, w/w (%)	0.26
Water content, w/w (%)	0.02

[a] The ratio is normalized per position and calculated as % of *sn-2* palmitic/3: of total palmitic acid x 100.

that derive mostly from high dietary intake of soybean and soybean products in China [16,17]. Despite this aforementioned diversity in the sn-1/3 position, the configuration of the palmitic acid in the mid position (*sn-2*) is highly conserved and characterized by a unique, highly specific, positional distribution on the glycerol backbone [6,18,19]. Previous studies have shown that this specific configuration of palmitic acid in the mid position reduces calcium salt formation leading to enhanced palmitic acid and calcium absorption as well as promoting other beneficial physiological functions [1,20–22].

As the TAGs composition of human milk plays a key role in infant health, the use of formula containing structured TAGs has gained much attention in recent years [13,20–25]. In this regard, INFAT® PLUS was recently introduced into the Chinese market and was designed to be closer in structure to Chinese human milk fat in all major TAGs species. INFAT® PLUS is a new grade of INFAT®, a vegetable oil blend that is commonly used in infant formulas (IF) and is Generally Recognized as Safe (GRAS) in the United States (U.S) [26]. INFAT® PLUS contains higher levels of OPL, a predominant C52 TAG found in Chinese human milk, and complies with the standard of 1,3-dioleoyl-2-palmitoyl-glycerol (OPO), known as *sn-2* palmitate fat [27]. OPO was previously shown to be associated with the improvement of fat and calcium absorption, bone health, intestinal microbiota, and infant comfort [28–32].

To confirm the safe consumption of INFAT® PLUS as an ingredient for infant formulas in the Chinese market, a series of safety studies were designed according to China's National Standards. The current study describes an acute toxicity test, battery of genotoxicity and mutagenicity tests, a 90-day oral toxicity test, and a teratology study. The results of these studies will provide the general toxicity properties of INFAT® PLUS including no-observed-adverse-effect Level (NOAEL) and the determination of genotoxicity and mutagenicity potential.

2. Materials and methods

2.1. Experimental design

The experimental procedures and protocols used in this study were designed and performed according to the Chinese Toxicology Assessment Procedures and Methods for Food Safety [33]. The Chinese National Food Safety Standard GB 15193.1-2014 was used to guide the design of the testing program, in the tests described below were those required by Chinese Regulatory Authorities (State Administration of Market Regulations (SAMR) in discussions with International Flavors & Fragrances. Section 6.4 of GB 15193.1-2014 states that for sensitive populations (e.g., infants), "*For foods consumed by pregnant women, lactating mothers or children, special attention should be paid to their embryotoxicity or reproductive developmental toxicity, neurotoxicity and immunotoxicity*." As an embryotoxicity study was conducted SAMR did not require reproductive development, neuro- or immunotoxicity studies.

2.2. Chemicals

All chemical reagents, solvents, pharmaceuticals, and other chemicals used in the studies were of analytical or pharmaceutical grade and were obtained from commercial sources.

2.3. Test materials

The compositional characteristics of INFAT® PLUS (Advanced Lipids AB) are presented in Table 1. The test ingredient used in the current study is comprised of 37.9 % palmitic acid of total fatty acids out of which 53.6 % esterified at the *sn-2* position of the triglyceride. The C52 TAG composition stands on 42.8 % of the total triglycerides, with an OPL to OPO ratio of 0.94. The characterization of INFAT® PLUS test substance was performed by trained personnel using established procedures and a validated method and was subjected to Quality Control review. The reference material was Soybean oil (CAS 8001–22–7) provided by Spectrum Chemical Mfg. Corp. (NJ, United States).

2.4. Dose formulation

INFAT® PLUS is a waxy solid material at room temperature with a density of 0.89 g/mL. Prior to daily use, INFAT® PLUS samples were warmed in a water bath incubator for 3 h at 65°C. Once the warmed material was visibly clear and homogeneous, INFAT® PLUS was diluted with soybean oil to create 50:50 and 20:80 stock mixtures. Under heated conditions (60°C-65°C), INFAT® PLUS and both stock mixtures were further divided into experimental samples. Syringes were pre-filled with the heated dosing solution and then allowed to cool at 30–33°C prior to oral gavage.

2.5. Animals

Experimental ICR mice and Sprague Dawley (SD) rats, certified specific pathogen-free (SPF), were provided by Zhejiang Experimental Animal Center under the license number SCXK (Zhe) 2019–0002. Animals were reared in the SPF animal house of Zhejiang Academy of Medical Sciences with license numbers SYXK (Zhe) 2019–0011 and SYXK (Zhe) 2016–0022 for both mice and rats, respectively. The animal room temperature range was 20–25 ℃, and the relative humidity range was 40–70 % with 12 h light/dark regime. Before commencing experiments, all animals were acclimatized to the laboratory environments for at least 3 days. During the experiment, all animals were provided purified water and solid feed (Zhejiang Experimental Animal Center) ad libitum. All animal experiments and procedures were approved by the Zhejiang Academy of Medical Sciences Ethics Committee (Acceptance No. ZJCLA-IACUC-20100008).

2.6. Acute oral toxicity

An acute toxicity test was performed according to the limit method in 20 ICR mice and 20 SD rats of both genders (10 females and 10 males per group) with body weight ranging from 18 to 22 g (~4 weeks) and 180–220 g (~6 weeks), respectively. The mice were fasted (water supplied ad libitum) for 6 h prior to gavage administration. The volume administrated by oral gavage was 20 mL/kg BW; due to the viscosity of the test material, administration was three times a day (TID) within an interval of 4 h, with a total dose of 53.4 mg/kg BW. Feed was supplied ad libitum 2 h after the last gavage dose was administered. SD rats were fasted (water supplied ad libitum) for 16 h prior to gavage administration. The volume administrated by oral gavage was 10 mL/kg BW, TID within an interval of 4 h, with a total dose of 26.7 mg/kg BW. Feed was supplied ad libitum 3 h after the last gavage dose was administered. During a 14-day period, all animals were monitored daily for mortality

or changes in clinical and behavioral signs. The body weight of each animal was recorded before dosing and on day 14. At the end of the observation period (day 15), all animals were sacrificed and subjected to gross pathological examinations.

2.7. Genetic toxicity

The genetic toxicity potential of INFAT® PLUS was evaluated in vitro in a bacterial reverse mutation (AMES) test and in vivo in a mammalian erythrocyte micronucleus test and a chromosome aberration test of mouse spermatogonia/spermatocyte. All studies were considered valid based on prespecified acceptance criteria stated in the applicable test guidelines.

2.7.1. Bacterial reverse mutation (AMES) test

INFAT® PLUS was examined for its possible mutagenic activity in the bacterial reverse mutation test using Histidine auxotrophic *Salmonella enterica* serovar typhimurium strains TA_{97a}, TA_{98}, TA_{100}, TA_{102}, TA_{1535} in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix). The strains were provided by Shanghai Municipal Center for Disease Control & Prevention and were tested after passing biological identification. As there was no cytotoxicity in a preliminary toxicity test with the non-metabolic activation of the TA100 strain, (5000) μg/plate was chosen as the maximum dose level with additional four dose levels of 1667, 556,185, 62 μg/plate. Furthermore, a verification test was performed, testing a different set of dose levels (8, 40, 200, 1000, 5000 μg/plate), keeping other conditions unchanged. The stock test solution (0.5 g INFAT® PLUS in 10 mL sterile DMSO) was serially diluted in sterilized DMSO to deliver the required concentration in a constant volume. The controls included a blank control, distilled water and DSMO (100 μL/plate). The positive controls were standard mutagens as follows, sodium azide (1.5 μg/plate) for TA_{100} and TA_{1535} without S9-mix, Dexon (50 μg/plate) for TA_{97a}, TA_{98}, and TA_{102} without S9-mix, 2-acetamidofluorene (10 μg/plate) for TA_{97a}, TA_{98}, and TA_{100} with S9-mix, 1,8- dihydroxyanthraquinone (25 μg/plate) for TA_{102} with S9-mix, and cyclophosphamide (200 μg/plate) for TA_{1535} with S9-mix. Three test plates per concentration were incubated at 37 °C for 48 h and then counted. The number of revertant colonies was counted manually for each plate.

2.7.2. Mammalian erythrocyte micronucleus test

INFAT® PLUS was evaluated in vivo for its ability to induce micronuclei in rodent bone marrow by analyzing peripheral blood reticulocytes (RETs) from ICR mice. Fifty mice weighing 25–30 g (~7 weeks), with half male and female, were randomly divided into five groups (10/group). Mice were treated by oral gavage (20 mL/kg BW with soybean oil (negative control), Cyclophosphamide (40 mg/kg BW; positive control) or INFAT® PLUS at 4.5, 8.9 or 17.9 g/kg BW. All groups were treated by oral gavage twice within 24 h. The mice were killed by cervical dislocation 6 h after the second gavage was finished. The sternum bone marrow was collected to make bone marrow slices, fixed with methanol, and stained with Giemsa. Red blood cells (RBC) and polychromatic erythrocytes (PCE) were observed under microscopy. Two hundred red blood cells from the bone marrow of each animal were evaluated, and the proportion of PCE out of total RBC was determined (PCE:RBC ratio). An additional 2000 PCE were examined per animal with the number of micronucleated PCE recorded to determine the incidence of micronucleus. The rates of micronucleus-containing cells in each group/sex were compared with the rates of micronucleus-containing cells in the negative control group.

2.7.3. Chromosome aberration test of mouse spermatogonia or spermatocyte

Twenty-five healthy adult male ICR mice were randomly divided into five groups (5/group, ~7 weeks)). The five groups included a negative control group (soybean oil), a positive control group (40 mg/kg BW cyclophosphamide), and 3 INFAT® PLUS treatment groups (4.5, 8.9 or 17.8 g/kg BW). All the mice were treated by oral gavage with volume of 20 mL/kg BW, once daily for 5 successive days. On the 12th day, all animals were injected with colchicine (6 mg/kg). After 4 h, the mice were killed by cervical dislocation technique and both testicles were excised and mixed with trisodium citrate (w/v, 1 %). Then, the seminiferous tubules were separated, fixed twice, and centrifuged at 1000 rpm for 10 min. After the supernatant was discarded, the remaining tissues was dried in air and stained with Giemsa. The mitotic cells were evaluated under a microscope at 100X magnification, and 100 metaphase cells were counted for each mouse for a total of 500 metaphase cells/group. The different types of chromosome structural aberrations in each group were recorded, and the cell aberration rate, monovalent chromosomes, and monovalent sex chromosome were determined.

2.8. 90-Day subchronic rodent feeding study

2.8.1. Study design

The subchronic toxicity test was performed in 130 healthy SD rats of both genders (female to male ratio 1:1) with an average body weight ranging from 60 to 80 g per the GB 15193.13 standard (50–100 g and <6 weeks of age). After 3 days of acclimatization, the rats were randomly assigned into five main groups (20 rats/group) and 3 satellite groups (10 rats/group). The main groups included a negative control group (distilled water), a solvent control group (soybean oil), and three INFAT® PLUS dose levels 2.23, 4.45, 8.9 g/kg BW/day. The highest dose level was established based on (A) the estimated infant formula consumption of 8.9 g INFAT® Plus/kg BW/day, and (B) animal welfare limits on dosing volume (GB standard of 4 mL/kg BW/day, which were exceeded in this study to provide a high dose level greater than potential human exposure). Mid- and low dose levels were selected as ½ the preceding dose to provide for a dose response analysis if necessary. The satellite groups included a solvent control group, a negative control group and a high-dose group. The test and control substances were prepared daily and administered via oral gavage at a dosing volume of 10 mL/kg BW. The duration of the experiment was 90 days whereas the satellite groups were treated for 45 days and sacrificed thereafter. The selected test substance dosing was calculated based on maximal IF consumption level of 260 mL/kg body weight/day recommended by EFSA Scientific Committee [34]. The high-dose group was designed to exceed the highest intake of INFAT® PLUS from IF (2.9 g/L), without compromising the nutritional value of animal's diet. The low and medium doses of were determined by a common ratio of two.

2.8.2. Clinical observations

During the treatment period, all animals were observed daily for general performance, signs of toxicity and mortality. Body weight and food consumption were measured weekly. The mean weekly body weight and food consumption utilization were calculated for each sex animal and dose level. Eye examination (cornea, conjunctiva, iris, crystalline lens) was performed in the solvent control group, negative control group and high-dose group before and at the end of the treatment period.

2.8.3. Urinalysis

Post-dose urine samples of individual rats were collected after fasting for 12 h (water provided ad libitum). Urinalysis was measured by GF-U180 urine analyzer (Shandong GaoMi Rainbow Analysis Instrument Co. Ltd., China) and included the determination of specific gravity (SG), pH, glucose (Glu), protein (PRO), and presence of blood (BLD).

2.8.4. Hematological and biochemical analysis

At the end of the treatment period and after night-fasting (not including water), rats were anesthetized by intraperitoneal injection of 8 % chloral hydrate with 0.5 mL/100 g BW. Blood samples were collected from abdominal aorta and used for hematological and biochemical

analysis. The whole blood was stabilized by the anticoagulant ethylene diamine tetraacetic acid (EDTA) and analyzed using an automatic TEK8530 blood analyzer (Jiangxi Tekang Technology Co. Ltd., China). The parameters evaluated included hemoglobin (HG), red blood cell counts (RBC), white blood cells count (WBC), percentages of lymphocyte (LYM%), percentages of granulocytes (GRA%), percentages of mid-range absolute count (MID%), platelet counts (PLT), and hematocrit (HCT). Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured by XL1800 blood coagulometer (Beijing Zonci Technology Development Co. Ltd., China). Clinical biochemistry parameters were analyzed using an automatic clinical analyzer Lan Yun LW C400 (Shenzhen Landwind Industry Co., Ltd., China) to determine alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (Alb), total cholesterol (TC), total triglyceride (TG), glucose (Glu), blood urea nitrogen (BUN), creatinine (CR), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), potassium (K), sodium (Na), and chloride (Cl).

2.8.5. Necroscopy and histopathology

At study termination, a complete gross necropsy was performed for main groups. During necropsy, the selected organs: brain, heart, liver, kidney, spleen, uterus/epididymis, ovaries/testis, thymus, and adrenal glands were isolated and weighted separately. The relative organ weights were calculated based on the fasted animal's body weight before anesthesia. Organs and tissues from each animal were fixed in 10 % formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin. Histopathological examinations were conducted on heart, liver, spleen, lung, kidney, brain, pituitary, thyroid, thymus, stomach, duodenum, pancreas, jejunum, ileum, colon, rectum, bladder, lymph nodes (mesenteric lymph nodes), adrenal glands, prostate, testis, epididymis, ovary, uterus of the solvent control group, negative control group and high-dose group.

2.9. Teratogenicity test

2.9.1. Animals and mating

One hundred females and thirty male SD rats, weighing 180–220 g, were used for the teratogenicity study; males were only used for mating, i.e., they were not exposed to the test material. After the acclimatization period, female and male rats were housed at a ratio of 2:1 overnight for mating. Successful mating was confirmed by examining for the presence of sperm on the vaginal smear in the morning. The day of positive vaginal smear or plug was considered day 0 of gestation (GD0). After positive evidence of mating, the males were returned to their separate cages. All animals were allowed to have free access to solid feed and drinking water.

2.9.2. Study design

Mated females were weighed and randomly assigned into 4 groups, with 19–20 rats in each group. The groups included a solvent control group (soybean oil), and INFAT® PLUS at doses of 2.23, 4.45, 8.9 g/kg BW/day. All rats were exposed daily for 10 consecutive days (from GD6-GD15) by oral gavage with a dosing volume of 10 mL/kg BW. On GD20, all female rats were sacrificed following euthanasia by 2,2,2-Tribromoethanol (Shanghai Aladdin Biochemical Technology Co., Ltd., China).

2.9.3. Experimental variables

Animals were observed daily for general clinical condition, behavior, and mortality. Body weights were recorded on GD0, 6, 9, 12, 15 and 20. At the end of the observation period, the uterus was removed and weighed, and the number of corpus luteum, percent of implantation, live and dead fetuses, stillbirths, and embryo/fetal absorptions was recorded. For live fetuses, fetal body length, weight, gender, and general appearance of teratogenicity were recorded. After examination and measurement, live fetuses from each litter were randomly divided into two groups for skeletal and visceral examination, respectively. For skeletal evaluation, fetuses from each litter were immersed in ethanol (v/v, 95 %) for 3 weeks, cleared in potassium hydroxide (w/w, 2 %) for 3 days, and then stained with Alizarin Red S for 2 days. For visceral examination, fetuses from each litter were immersed in Bouin's solution for 2 weeks to observe visceral abnormalities.

Table 2
Results of acute toxicity study INFAT® Plus.

ICR mice				
Sex	Starting weight (g)	Final weight (g)*	Deaths	LD_{50} (g/kg)
Female	19.9 ± 1.4	30.3 ± 1.3	0/10	>53.4
Male	19.7 ± 1.3	31.5 ± 2.3	0/10	>53.4
SD rats				
Sex	Starting weight (g)	Final weight (g)**	Deaths	LD_{50} (g/kg)
Female	198.4 ± 13.6	236.3 ± 11.2	0/10	>26.7
Male	200.5 ± 13.1	312.8 ± 15.1	0/10	>26.7

* Normal weight range for ICR mice at 6 weeks is ~25–35 g. [46]
** Normal weight range for Sprague-Dawley rats at 8 weeks is ~240–320 g (male) and ~160–240 g (female). [47]

2.9.4. Statistical analysis

Statistical analysis of the experimental data was conducted by comparing the tested groups with control groups using SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Continuous variables were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). If the variance was not uniform, appropriate transformation of the original data was performed once, and the transformed data was re-evaluated for homogeneity of variance. In the case of homogeneous variance, the data were subjected to one-way analysis of variance (ANOVA), otherwise, data were analyzed by a rank sum test. If either of the tests showed significant differences, Dunnett's test (uniform variance) or Tamhane's T2 test (non-uniform variance) was used for multiple comparisons. Categorized variables were subjected to Chi-square and Fisher's exact test. Statistical significance was defined as the p-values less than 0.05 ($p < 0.05$).

3. Results

3.1. Acute oral toxicity

In the single dose toxicity study, oral gavage administration of INFAT® PLUS at 53.4 g /kg BW in mice and 26.7 g/kg BW in rats was well tolerated. No death was recorded in any of the groups during 14 days of the study, and no clinical signs of toxicity related to the administration of INFAT® PLUS were observed. Food consumption and water intake did not indicate any treatment-related adverse effects, and body weight gain of the animals was within normal range (Table 2). The necropsy at the end of study did not reveal any gross pathological abnormalities. Hence, the oral median lethal dose (LD50) values of INFAT® PLUS were determined to be greater than 53.4 g /kg BW and 26.7 g/kg BW for ICR mice and SD rats, respectively.

3.2. Genetic toxicity

3.2.1. Bacterial reverse mutagenicity (AMES) test

The results of the bacterial reverse mutation assay in the presence or absence of metabolic activation are shown in Table 3. No cytotoxicity was observed and all positive control mutagens clearly increased the number of revertant colonies (~3–100-fold) under the tested conditions. The mean numbers of revertant colonies counted in the INFAT® PLUS treated groups (up to 5000 μg/plate) were similar to those of the blank control, regardless of the presence of metabolic activation. Moreover, the confirmatory test showed similar results where INFAT® PLUS did not induce any dose-related increases in the mean number of revertant colonies.

Table 3
Results of bacterial reverse mutation (Ames) test for INFAT® PLUS.

Substance	TA_{97a}		TA_{98}		TA_{100}		TA_{102}		TA_{1535}	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Main test										
Blank control	114.0 ± 12.2	144.7 ± 13.0	31.0 ± 4.4	42.7 ± 3.8	134.3 ± 13.1	161.7 ± 16.1	253.7 ± 22.0	292.3 ± 19.9	13.3 ± 2.5	15.3 ± 2.1
Distilled water	125.3 ± 7.0	139.3 ± 15.3	31.7 ± 2.1	45.0 ± 5.6	150.3 ± 13.6	168.7 ± 18.9	256.0 ± 20.0	303.7 ± 19.1	12.0 ± 1.7	15.3 ± 2.3
DMSO 100 µL/plate	121.3 ± 21.2	151.3 ± 22.4	34.7 ± 5.0	44.3 ± 5.1	143.7 ± 21.1	171.3 ± 20.1	261.3 ± 15.3	301.7 ± 20.2	12.0 ± 3.6	14.7 ± 3.5
INFAT®PLUS										
62 µg/ plate	111.7 ± 15.9	140.3 ± 12.7	32.0 ± 3.6	41.3 ± 4.2	130.7 ± 13.6	164.0 ± 17.1	248.7 ± 20.2	293.3 ± 18.6	11.7 ± 1.5	14.7 ± 2.3
185 µg/ plate	103.7 ± 9.0	136.7 ± 9.5	31.3 ± 4.0	35.3 ± 4.2	125.3 ± 12.9	156.7 ± 16.5	247.0 ± 16.5	301.7 ± 21.8	13.3 ± 2.1	14.3 ± 3.8
556 µg/ plate	115.0 ± 13.5	151.0 ± 14.9	33.3 ± 4.2	39.7 ± 3.2	138.3 ± 21.5	158.3 ± 9.9	252.7 ± 19.4	284.7 ± 16.8	13.7 ± 3.2	12.7 ± 2.5
1667 µg/ plate	120.7 ± 21.0	138.7 ± 20.4	30.7 ± 4.6	42.0 ± 4.0	136.3 ± 9.5	171.7 ± 14.6	257.3 ± 16.0	294.3 ± 18.9	12.0 ± 3.6	14.7 ± 2.5
5000 µg/ plate	112.3 ± 14.3	149.3 ± 12.7	34.7 ± 4.7	44.3 ± 4.0	141.0 ± 12.1	168.7 ± 21.1	261.7 ± 15.6	299.0 ± 15.4	12.0 ± 1.7	12.3 ± 2.1
Positive control[a,b]	2480.7 ± 94.3	1696.7 ± 145.7	1153.3 ± 164.4	4833.3 ± 195.5	2726.7 ± 150.1	3060.0 ± 185.2	856.7 ± 130.1	926.7 ± 68.1	1246.7 ± 90.7	271.3 ± 16.0
Verification test										
Blank control	115.7 ± 11.9	142.3 ± 20.8	32.3 ± 3.2	43.3 ± 4.0	131.3 ± 14.7	162.7 ± 22.0	254.7 ± 22.0	292.3 ± 15.6	11.7 ± 1.5	17.7 ± 7.5
Distilled water	121.3 ± 13.0	163.3 ± 6.1	31.7 ± 2.5	43.0 ± 4.4	137.0 ± 9.5	172.7 ± 7.0	248.3 ± 11.1	316.7 ± 13.0	10.3 ± 3.2	14.3 ± 3.2
DMSO	122.3 ± 21.5	152.0 ± 15.6	34.7 ± 4.5	45.7 ± 3.5	143.7 ± 20.6	172.3 ± 15.3	262.3 ± 15.6	302.7 ± 21.4	13.0 ± 2.0	14.3 ± 2.1
INFAT®PLUS										
8 µg/ plate	113.3 ± 9.0	146.3 ± 20.6	30.7 ± 3.1	42.3 ± 4.9	130.3 ± 13.1	163.7 ± 15.2	254.0 ± 20.9	294.0 ± 18.0	12.7 ± 2.5	11.7 ± 3.1
40 µg/ plate	107.7 ± 15.1	150.3 ± 13.7	32.3 ± 3.5	35.7 ± 4.7	127.0 ± 9.6	161.7 ± 12.7	246.7 ± 18.5	300.7 ± 20.3	13.3 ± 3.8	14.3 ± 3.8
200 µg/ plate	114.0 ± 20.9	135.0 ± 9.5	31.7 ± 4.7	39.3 ± 4.2	131.3 ± 13.7	157.3 ± 21.4	251.7 ± 12.7	285.3 ± 21.6	12.3 ± 2.1	15.3 ± 3.1
1000 µg/ plate	119.3 ± 12.2	149.3 ± 15.5	33.0 ± 3.6	41.0 ± 5.6	134.7 ± 20.2	164.7 ± 9.0	258.7 ± 16.5	297.3 ± 18.6	14.3 ± 2.1	12.7 ± 1.5
5000 µg/ plate	121.3 ± 13.3	151.7 ± 15.2	34.7 ± 5.1	43.7 ± 4.0	142.7 ± 16.3	172.0 ± 14.7	261.3 ± 17.9	302.3 ± 20.1	12.0 ± 3.0	16.7 ± 1.5
Positive control[a,b]	2493.3 ± 245.8	1596.7 ± 172.4	1166.7 ± 72.3	4553.3 ± 438.8	2736.7 ± 111.5	2960.0 ± 255.1	866.7 ± 83.3	943.3 ± 40.4	1256.7 ± 175.0	246.3 ± 10.5

Data expressed as mean ± standard deviation of revertant colonies in triplicate assay systems; + S9: with S9-mix; - S9: without S9-mix; [a]Positive controls without S9-mix: Dexon (50 µg/plate) for TA97a, TA98, and TA102; sodium azide (1.5 µg/plate) for TA100 and TA153; [b]Positive controls with S9-mix: 2-acetamidofluorene (10 µg/plate) for TA97a, TA98, and TA100; 1,8- dihydroxyanthraquinone (25 µg/plate) for TA102;cyclophosphamide (200 µg/plate) for TA1535.

Table 4
Results of in vitro mammalian erythrocyte micronucleus test for INFAT®PLUS.

Sex	Groups	Dose	No. of mice examined	Micronucleus analysis		PCE analysis	
				Number of PCE examined per animal	MNPCE rate (‰)	Number of RBC examined per animal	PCE/RBC (%)
Female	Negative control	—	5	2000	2.8 ± 0.3	200	51.8 ± 0.67
	INFAT® PLUS	4.5 g/kg BW	5	2000	2.7 ± 0.6	200	51.7 ± 0.57
		8.9 g/kg BW	5	2000	3.1 ± 0.5	200	51.9 ± 0.54
		17.8 g/kg BW	5	2000	2.9 ± 0.4	200	51.9 ± 0.65
	Cyclophosphamide	40 mg/kg	5	2000	22.9 ± 0.7 *	200	48.3 ± 0.57
Male	Negative control	—	5	2000	2.9 ± 0.4	200	51.8 ± 0.57
	INFAT® PLUS	4.5 g/kg BW	5	2000	2.8 ± 0.4	200	51.7 ± 0.57
		8.9 g/kg BW	5	2000	3.0 ± 0.6	200	51.7 ± 0.45
		17.8 g/kg BW	5	2000	3.2 ± 0.6	200	51.5 ± 0.61
	Cyclophosphamide	40 mg/kg	5	2000	22.8 ± 1.3 *	200	48.4 ± 0.65

Data expressed as mean ± standard deviation for 5 mice; Negative control: soybean oil; Positive control: cyclophosphamide; MNPCE: micronucleated polychromatic erythrocytes; RBC: Red blood cells; PCE: polychromatic erythrocytes; *$p < 0.05$ compared with control solvent.

3.2.2. *Mammalian erythrocyte micronucleus test*

Table 4 presents the findings of the mammalian erythrocyte micronucleus test. There were no statistically significant differences in the PCE/RBC ratio and the micronucleus rate between INFAT® PLUS at any dose tested (4.5, 8.9 or 17.9 g/kg BW) vs. negative control group ($p > 0.05$). All PCE/RBC changes were within 20 % of the negative control group, with no observed signs of cytotoxicity. In contrast, the rate of micronucleus containing PCE in the cyclophosphamide treated group was significantly higher than that in the negative control group ($p < 0.01$), confirming the validity of the test.

Table 5
Results of In vivo chromosome aberration test of mouse spermatogonia or spermatocyte for INFAT®PLUS.

Groups	Dose	No. of cells examined	Type of chromosome structural aberrations, n (%)†			Abnormal chromosomal cells, n (%)†
			Sex chromosome monovalent body	Autosomal monovalent body	Chromosome aberration	
Negative control	—	500	0 (0.0)	4 (0.8)	7 (1.4)	6 (1.2)
INFAT®PLUS	4.5 g/kg BW	500	0 (0.0)	2 (0.4)	5 (1.0)	3 (0.6)
	8.9 g/kg BW	500	0 (0.0)	3 (0.6)	6 (1.2)	4 (0.8)
	17.8 g/kg BW	500	0 (0.0)	2 (0.4)	5 (1.0)	4 (0.8)
Cyclophosphamide	40 mg/kg	500	0 (0.0)	14 (2.8)**	72 (14.4)**	52 (10.4)**

Negative control: soybean oil; †Rate (%) is calculated based on number of items observed divided by 500 cells examined x 100 %; **$p < 0.01$ compared with negative control.

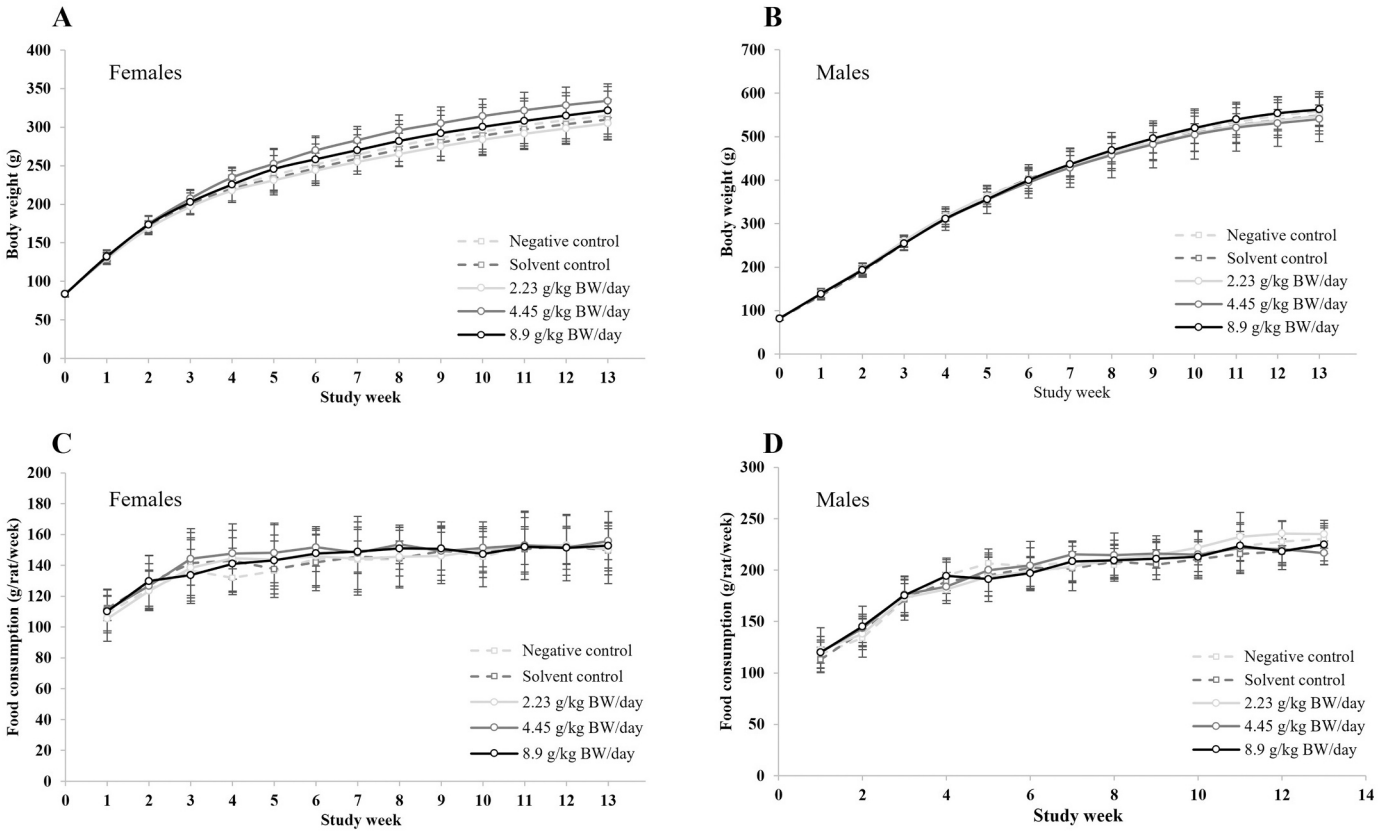

Fig. 1. Body weight (A-B; 10 rats/sex/group) and weekly food consumption (C-D; 5 rats/sex/group) of rats in the 90-day subchronic feeding study. The values are presented as means ± standard deviation. No significant differences were observed.

3.2.3. Chromosome aberration test of mouse spermatogonia or spermatocyte

Compared with the negative control group, INFAT® PLUS at all dose levels had no significant differences ($p > 0.05$) in the rate of chromosomal abnormal cells (Table 5). Similarly, no statistical differences were observed with the total count of chromosome aberration, autosomal bodies, and sex chromosome monovalent bodies between INFAT® PLUS treated groups and negative control group. On the other hand, the number of autosomal monovalent body, chromosome aberration, and the number of abnormal chromosomal cells, as well as the rate of abnormal chromosomal cells in the cyclophosphamide treated group was significantly higher compared with the negative control group ($p < 0.01$).

3.3. 90-Day subchronic rodent feeding study

3.3.1. Clinical observations, body weight and food consumption

All animals survived to scheduled sacrifice. There were no relevant clinical signs, or abnormal ophthalmological findings noticed in either the control or treated rats throughout the 90-day experimental period. No significant differences were observed in body weight, or food consumption between rats in any dose level in comparison to their control groups (Fig. 1). Similarly, food efficiency was not affected by INFAT® PLUS treatment (data not shown).

3.3.2. Hematology, coagulation, blood chemistry and urinalysis

There were no test item-related biologically significant adverse effects observed in hematology/coagulation (Table 6), blood chemistry (Table 7) or urinalysis (data not shown) for both sexes across the groups in either the main study, or the interim satellite group (data not shown). Occasional, statistically significant, differences were noted between the medium-dose group, high-dose group vs. negative control group, however, all values of the treated groups were within the normal reference value range, so these changes were not considered to have toxicological significance.

Table 6
Hematological/coagulation values of rats treated orally with INFAT® PLUS for 90 days.

Sex	Item	Negative Control	Solvent Control	INFAT® PLUS dose level		
				2.23 g/kg BW	4.45 g/kg BW	8.9 g/kg BW
Female	HG (g/L)	139.9 ± 5.0	141.3 ± 5.3	141.7 ± 4.1	141.7 ± 5.8	144.6 ± 5.4
	RBC ($\times 10^{12}$/L)	6.90 ± 0.19	6.78 ± 0.28	6.90 ± 0.22	6.83 ± 0.36	6.91 ± 0.34
	WBC ($\times 10^{9}$/L)	3.9 ± 1.1	3.0 ± 1.1	3.6 ± 0.9	4.0 ± 1.3	3.3 ± 0.8
	LYM (%)	68.0 ± 4.9	68.1 ± 7.1	67.3 ± 5.4	67.9 ± 6.6	68.3 ± 3.3
	GRA (%)	16.9 ± 2.1	18.1 ± 3.4	15.2 ± 3.7	17.7 ± 4.9	16.2 ± 2.6
	MID (%)	15.1 ± 4.2	13.9 ± 4.3	17.5 ± 3.6	14.5 ± 4.1	15.5 ± 3.0
	PLT ($\times 10^{9}$/L)	557.9 ± 58.3	535.4 ± 46.4	566.2 ± 83.7	579.1 ± 56.0	581.6 ± 59.9
	HCT (%)	32.1 ± 1.2	30.9 ± 1.3	31.8 ± 0.8	31.5 ± 1.4	31.5 ± 1.5
	APTT (s)	13.1 ± 1.5	13.4 ± 1.2	13.7 ± 1.0	14.1 ± 0.4	14.0 ± 1.7
	PT (s)	10.3 ± 0.3	10.1 ± 0.5	10.1 ± 0.4	10.2 ± 1.3	10.7 ± 1.3
Male	HG (g/L)	154.8 ± 9.3	152.1 ± 5.5	150.7 ± 4.3	153.0 ± 6.4	151.7 ± 6.2
	RBC ($\times 10^{12}$/L)	7.33 ± 0.60	7.39 ± 0.34	7.55 ± 0.26	7.57 ± 0.34	7.57 ± 0.25
	WBC ($\times 10^{9}$/L)	7.4 ± 1.4	6.8 ± 2.4	6.9 ± 2.0	6.0 ± 1.6	6.2 ± 1.8
	LYM (%)	62.8 ± 6.5	58.4 ± 6.9	58.0 ± 6.3	56.4 ± 6.9	56.9 ± 7.8
	GRA (%)	21.5 ± 5.0	24.1 ± 5.9	24.4 ± 4.2	24.7 ± 5.1	23.9 ± 5.1
	MID (%)	15.8 ± 5.9	17.5 ± 2.9	17.7 ± 3.8	19.0 ± 4.9	19.2 ± 3.7
	PLT ($\times 10^{9}$/L)	542.1 ± 44.9	575.6 ± 69.3	571.8 ± 57.8	575.7 ± 48.4	549.6 ± 61.2
	HCT (%)	32.9 ± 2.4	33.1 ± 0.9	33.5 ± 1.3	33.6 ± 1.6	33.7 ± 1.6
	APTT (s)	14.1 ± 1.2	14.4 ± 1.1	15.1 ± 0.9	14.5 ± 1.6	14.9 ± 0.7
	PT (s)	11.2 ± 1.2	12.0 ± 1.6	11.4 ± 1.0	10.7 ± 0.5	12.1 ± 1.7

Data expressed as mean ± standard deviation for 10 rats per sex group; L: liter; g: gram; s: second; HG: hemoglobin; RBC: red blood cell counts; WBC: white blood cells count; LYM%: percentages of lymphocyte; GRA%: percentages of granulocytes; MID%: percentages of mid-range absolute count; PLT: platelet counts; HCT: hematocrit; APTT: Activated partial thromboplastin time; PT: prothrombin time; No significant differences were observed.

3.3.3. Organ weights, gross pathology, and histopathology

Organ weights (absolute and relative) data are summarized in Table 8. There were no significant ($p > 0.05$) differences on any of the organ weights comparing with control groups at termination of the experimental period for either gender. At necropsy, there were no macroscopic abnormalities in the color, size, morphology, and structure of the tissues/organs evaluated. Similarly, no treatment-related histopathological findings were noted in the high-dose group as compared to the control groups. All findings observed were consistent with normal background lesions in rats of this age and were considered spontaneous and/or incidental in nature and unrelated to INFAT® PLUS administration.

3.4. Teratology study

During the study, no treatment-related clinical signs of toxicity or mortality were observed in the pregnant rats, irrespective of treatment

Table 7
Blood chemistry values of rats treated orally with INFAT® PLUS for 90 days.

Sex	Item	Negative control	Solvent control	INFAT® PLUS dose level		
				2.23 g/kg BW	4.45 g/kg BW	8.9 g/kg BW
Female	ALT (U/L)	46.4 ± 7.9	43.8 ± 13.5	46.2 ± 6.6	48.5 ± 14.5	48.0 ± 9.1
	AST (U/L)	124.9 ± 20.3	114.5 ± 35.4	105.4 ± 14.6	117.9 ± 26.6	108.5 ± 24.8
	TP (g/L)	67.6 ± 3.2	65.9 ± 2.8	65.0 ± 1.2	66.6 ± 2.8	65.7 ± 3.7
	Alb (g/L)	35.5 ± 3.0	36.2 ± 1.9	33.5 ± 1.7	36.2 ± 1.7	35.7 ± 2.2
	TC (mmol/L)	2.44 ± 0.35	2.09 ± 0.38	2.03 ± 0.29 *	2.21 ± 0.40	2.10 ± 0.36
	TG (mmol/L)	0.36 ± 0.15	0.33 ± 0.14	0.32 ± 0.15	0.30 ± 0.14	0.28 ± 0.12
	Glu (mmol/L)	6.24 ± 0.90	6.61 ± 0.86	6.77 ± 0.61	6.77 ± 0.91	6.45 ± 0.59
	BUN (mmol/L)	6.61 ± 0.94	5.56 ± 0.63	5.89 ± 1.34	6.79 ± 1.75	6.49 ± 0.74
	CR (μmol/L)	56.3 ± 1.9	55.7 ± 2.1	52.9 ± 2.8 *†	56.0 ± 2.1	57.5 ± 2.4
	GGT (U/L)	9.1 ± 0.6	9.9 ± 1.9	9.8 ± 0.7	9.6 ± 0.7	9.2 ± 1.2
	ALP (U/L)	52.7 ± 12.7	51.9 ± 14.0	61.6 ± 25.0	63.7 ± 13.2	60.7 ± 14.7
	K (mmol/L)	4.31 ± 0.40	4.33 ± 0.26	4.40 ± 0.31	4.60 ± 0.33	4.32 ± 0.27
	Na (mmol/L)	139.3 ± 2.8	139.7 ± 2.3	139.8 ± 2.6	139.6 ± 2.8	139.9 ± 2.9
	Cl (mmol/L)	107.0 ± 2.0	108.1 ± 1.7	107.6 ± 1.8	108.1 ± 1.4	108.2 ± 1.5
Male	ALT (U/L)	68.7 ± 17.7	62.0 ± 9.0	61.4 ± 9.3	54.2 ± 13.5 *	60.8 ± 7.8
	AST (U/L)	162.8 ± 30.7	149.9 ± 24.5	151.9 ± 24.2	146.6 ± 22.9	126.4 ± 22.2 *
	TP (g/L)	62.4 ± 2.1	60.6 ± 2.1	61.2 ± 1.9	59.5 ± 2.2 *	61.7 ± 2.9
	Alb (g/L)	32.2 ± 1.0	32.7 ± 2.8	32.0 ± 1.0	32.1 ± 4.4	32.3 ± 1.2
	TC (mmol/L)	1.85 ± 0.21	1.96 ± 0.30	1.88 ± 0.22	1.67 ± 0.29†	1.79 ± 0.20
	TG (mmol/L)	0.43 ± 0.11	0.40 ± 0.19	0.36 ± 0.16	0.33 ± 0.15	0.34 ± 0.12
	Glu (mmol/L)	6.83 ± 1.56	7.62 ± 1.30	7.56 ± 1.41	7.58 ± 1.61	7.97 ± 0.81
	BUN (mmol/L)	6.28 ± 1.10	5.35 ± 0.86	5.44 ± 0.87	4.95 ± 0.54 *	6.06 ± 0.97
	CR (μmol/L)	56.2 ± 2.9	57.0 ± 2.4	57.2 ± 3.4	56.3 ± 5.2	57.9 ± 2.4
	GGT (U/L)	6.4 ± 1.7	6.1 ± 1.7	4.9 ± 1.9	6.0 ± 1.4	6.3 ± 2.5
	ALP (U/L)	122.1 ± 24.9	112.0 ± 36.3	101.8 ± 21.7	100.9 ± 26.8	103.6 ± 20.6
	K (mmol/L)	5.05 ± 0.26	5.30 ± 0.77	5.06 ± 0.38	5.03 ± 0.27	5.08 ± 0.26
	Na (mmol/L)	139.2 ± 2.1	138.5 ± 2.3	139.0 ± 2.1	139.7 ± 1.9	139.7 ± 1.9
	Cl (mmol/L)	104.9 ± 2.3	104.4 ± 3.1	105.1 ± 1.9	106.1 ± 1.9	105.9 ± 1.5

Data expressed as mean ± standard deviation for 10 rats per sex group; U: unit; L: liter; g: gram; mmol: millimole; μmol: micromole; ALT: alanine aminotransferase; AST; alanine aminotransferase; TP: total protein; Alb: albumin; TC: total cholesterol; TG: total triglyceride; Glu: glucose; BUN: blood urea nitrogen; CR: creatinine; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase; K: potassium; Na: sodium; Cl: chloride; *P < 0.05, compared with the negative control group; †P < 0.05, compared with the solvent control group.

or solvent control groups. There were no statistically significant differences in body weight (Fig. 2) or overall body weight gain (data not shown) in rats treated with INFAT® PLUS at any dose level in comparison to solvent control group. The effects of INFAT® PLUS on mated dams and fetuses are summarized in Table 9. Concerning maternal reproductive parameters, no statistically significant ($p > 0.05$) differences were observed in the rates of conceived rats, live fetuses, corpora lutea, implantation, pre-implantations mortality, absorbed births, stillbirth, gravid uterine weights, or sex ratios. Furthermore, maternal INFAT® PLUS exposure did not produce any adverse effects on GD 20 fetal weight, fetal body length, external, visceral, or skeletal morphology. In terms of skeletal examination, sternum defects and enlarged fontanelle were recorded; however, no significant ($p > 0.05$) differences were noted between the treated and solvent control groups.

4. Discussion

In the present study, a series of toxicological tests on INFAT® PLUS was performed, including acute toxicity, subchronic (90-day oral) toxicity, genotoxicity, and teratogenicity tests. All the tests provided a comprehensive safety assessment for INFAT®PLUS, with a primary line of comparison to soybean oil, a related oil that is commonly used in commercial infant formulas [35].

In the acute phase, the safety data of INFAT® PLUS was used to assess its hazard classification that may arise from acute oral exposure [36]. Based on the data presented and in accordance with the provision of acute toxicity test in Chinese Procedures for Toxicological Assessment of Food, INFAT® PLUS was assigned to the lowest toxicity class (>5 g /kg BW) and regarded as non-toxic [33].

Information on genotoxicity is another key component as part of the toxicological assessment of food [33]. In this sense, the safety of INFAT® PLUS was further demonstrated by a battery of in vitro and in vivo studies including bacterial reverse mutation (Ames) test, mouse erythrocyte micronucleus test, and chromosome aberration test of mouse spermatogonia/spermatocyte. Overall, the results of the three assays demonstrate that INFAT® PLUS does not cause DNA or chromosomal damage, confirming that INFAT® PLUS was not genotoxic.

To further assess the safety of INFAT®PLUS, repeated dose toxicity study was performed to evaluate possible adverse effects that may arise from repeated exposure of INFAT® PLUS over a prolonged period. Per regulatory guidelines [33], a 90-day repeated oral toxicity study was performed in SD rats with the high-dose group designed to exceed the highest intake of INFAT® PLUS from IF (2.9 g/L), without compromising the nutritional value of animal's diet, and for consistency with margin of exposure (≥ 1) of other macro-additive substances used in IF

Table 8
Absolute and relative organ weights of rats treated orally with INFAT® PLUS for 90 days.

Sex	Parameter	Weight	Negative control	Solvent control	INFAT® PLUS dose level		
					2.23 g/kg BW	4.45 g/kg BW	8.9 g/kg BW
Female	Terminal FBW	Absolute (g)	291.7 ± 29.3	285.0 ± 24.5	284.0 ± 20.5	310.9 ± 22.2	296.0 ± 26.9
	Brain	Absolute (g)	1.484 ± 0.091	1.434 ± 0.087	1.443 ± 0.037	1.457 ± 0.082	1.436 ± 0.056
		Relative (%)	0.51 ± 0.04	0.51 ± 0.06	0.51 ± 0.04	0.47 ± 0.02	0.49 ± 0.04
	Heart	Absolute (g)	0.999 ± 0.143	0.941 ± 0.105	0.955 ± 0.081	0.983 ± 0.162	0.991 ± 0.118
		Relative (%)	0.344 ± 0.050	0.330 ± 0.026	0.337 ± 0.019	0.316 ± 0.047	0.335 ± 0.032
	Liver	Absolute (g)	8.24 ± 0.81	8.37 ± 0.68	8.22 ± 0.71	9.07 ± 0.92	8.55 ± 0.64
		Relative (%)	2.84 ± 0.33	2.94 ± 0.17	2.90 ± 0.18	2.91 ± 0.18	2.89 ± 0.17
	Kidney	Absolute (g)	2.16 ± 0.23	2.02 ± 0.26	2.07 ± 0.23	2.21 ± 0.30	2.19 ± 0.05
		Relative (%)	0.74 ± 0.09	0.71 ± 0.05	0.73 ± 0.06	0.72 ± 0.12	0.74 ± 0.04
	Spleen	Absolute (g)	0.520 ± 0.105	0.499 ± 0.111	0.520 ± 0.067	0.539 ± 0.080	0.544 ± 0.041
		Relative (%)	0.180 ± 0.038	0.175 ± 0.032	0.184 ± 0.032	0.174 ± 0.025	0.184 ± 0.016
	Uterus	Absolute (g)	0.62 ± 0.11	0.64 ± 0.15	0.64 ± 0.19	0.60 ± 0.16	0.68 ± 0.23
		Relative (%)	0.21 ± 0.03	0.23 ± 0.06	0.22 ± 0.06	0.19 ± 0.04	0.23 ± 0.09
	Ovaries	Absolute (g)	0.148 ± 0.035	0.152 ± 0.019	0.153 ± 0.027	0.163 ± 0.036	0.149 ± 0.041
		Relative (%)	0.052 ± 0.016	0.054 ± 0.009	0.054 ± 0.011	0.053 ± 0.011	0.050 ± 0.012
	Thymus	Absolute (g)	0.346 ± 0.081	0.352 ± 0.126	0.364 ± 0.032	0.363 ± 0.053	0.372 ± 0.073
		Relative (%)	0.120 ± 0.030	0.125 ± 0.051	0.129 ± 0.012	0.117 ± 0.019	0.125 ± 0.021
	Adrenal glands	Absolute (g)	0.078 ± 0.017	0.088 ± 0.024	0.074 ± 0.011	0.079 ± 0.012	0.081 ± 0.016
		Relative (%)	0.027 ± 0.007	0.031 ± 0.009	0.026 ± 0.004	0.026 ± 0.005	0.028 ± 0.005
Male	Terminal FBW	Absolute (g)	513.5 ± 44.5	520.9 ± 43.0	524.3 ± 26.0	515.9 ± 53.0	534.9 ± 37.0
	Brain	Absolute (g)	1.534 ± 0.051	1.532 ± 0.078	1.573 ± 0.058	1.549 ± 0.041	1.563 ± 0.089
		Relative (%)	0.30 ± 0.03	0.30 ± 0.03	0.32 ± 0.02	0.30 ± 0.03	0.29 ± 0.02
	Heart	Absolute (g)	1.503 ± 0.128	1.625 ± 0.102	1.521 ± 0.139	1.544 ± 0.121	1.600 ± 0.114
		Relative (%)	0.295 ± 0.036	0.313 ± 0.015	0.290 ± 0.020	0.302 ± 0.035	0.300 ± 0.030
	Liver	Absolute (g)	14.11 ± 1.07	15.15 ± 2.26	14.46 ± 0.69	14.45 ± 2.05	15.27 ± 1.78
		Relative (%)	2.76 ± 0.22	2.90 ± 0.26	2.76 ± 0.13	2.80 ± 0.25	2.85 ± 0.19
	Kidney	Absolute (g)	3.46 ± 0.31	3.60 ± 0.35	3.36 ± 0.31	3.51 ± 0.39	3.57 ± 0.39
		Relative (%)	0.68 ± 0.05	0.69 ± 0.04	0.64 ± 0.05	0.68 ± 0.05	0.67 ± 0.07
	Spleen	Absolute (g)	0.802 ± 0.092	0.811 ± 0.234	0.763 ± 0.113	0.702 ± 0.103	0.760 ± 0.167
		Relative (%)	0.157 ± 0.018	0.157 ± 0.050	0.146 ± 0.020	0.136 ± 0.016	0.141 ± 0.025
	Testis	Absolute (g)	3.450 ± 0.425	3.540 ± 0.353	3.555 ± 0.316	3.452 ± 0.379	3.506 ± 0.135
		Relative (%)	0.67 ± 0.07	0.68 ± 0.06	0.68 ± 0.05	0.67 ± 0.07	0.66 ± 0.05
	Epididymis	Absolute (g)	1.44 ± 0.12	1.53 ± 0.30	1.45 ± 0.10	1.44 ± 0.19	1.39 ± 0.09
		Relative (%)	0.28 ± 0.02	0.29 ± 0.05	0.28 ± 0.02	0.28 ± 0.04	0.26 ± 0.02
	Thymus	Absolute (g)	0.473 ± 0.086	0.459 ± 0.128	0.444 ± 0.099	0.421 ± 0.097	0.446 ± 0.092
		Relative (%)	0.092 ± 0.016	0.088 ± 0.024	0.085 ± 0.020	0.083 ± 0.024	0.083 ± 0.017
	Adrenal glands	Absolute (g)	0.073 ± 0.020	0.079 ± 0.023	0.065 ± 0.011	0.067 ± 0.007	0.076 ± 0.014
		Relative (%)	0.014 ± 0.003	0.015 ± 0.005	0.012 ± 0.002	0.013 ± 0.002	0.014 ± 0.003

Data expressed as mean ± standard deviation for 10 rats per sex group; FBW: fasting body weight; g: gram; Relative%: organ weight/terminal FBW x 100 %; No significant differences were observed.

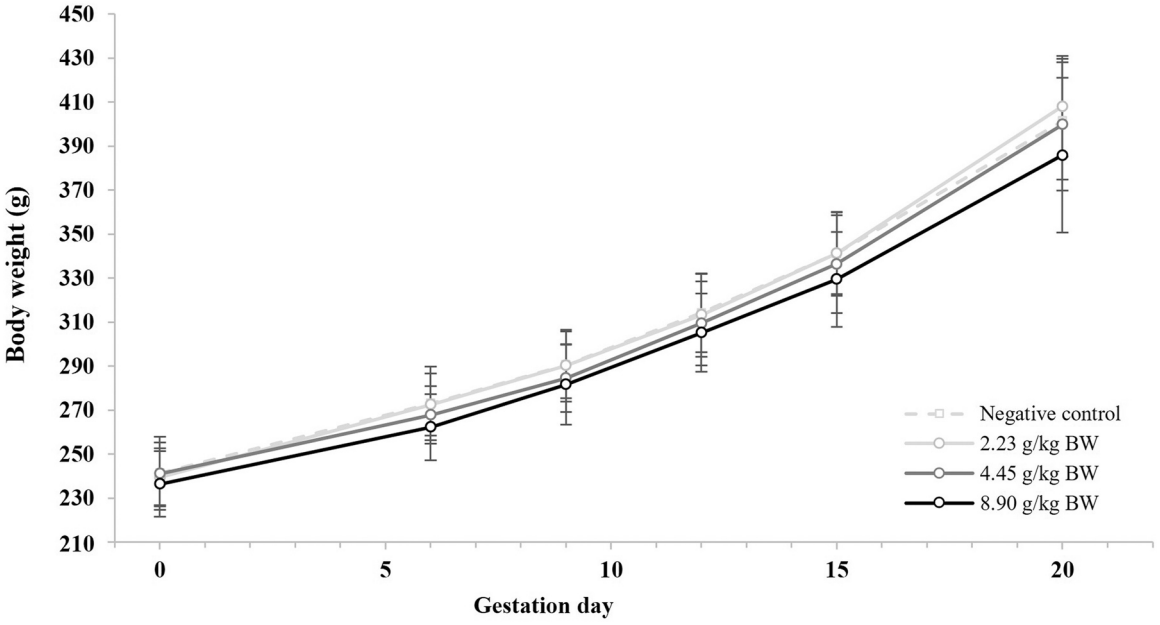

Fig. 2. Body weight of female rats during gestation period. The values are presented as means ± standard deviation. Each group included 16–17 conceived rats. No significant differences were observed.

[37–40]. While no major signs of toxicity were observed, occasional statistically significant differences were observed between INFAT® PLUS -treated groups and solvent control in some blood chemistry parameters, however these changes were all within the normal range of the laboratory historical reference and were considered unrelated to INFAT® PLUS INFAT® PLUS administration. Based on the results of the current study, the NOAEL of INFAT® PLUS in rats was set to be 8.90 g/kg BW/day. This value is consistent with previous observations, both in toxicological and human studies, reported on *sn-2* palmitate vegetable oil used in IF [26], and high levels of TAGs containing palmitic acid esterified at *sn-2* position that are found naturally in human milk [2, 6,19].

Additionally, a teratogenic study was carried out to evaluate the potential toxicity of INFAT® PLUS on the embryo/fetal development of SD rats. The NOAEL of INFAT® PLUS in the teratogenicity study was determined at the highest dose tested (i.e., 8.9 g/kg BW), which was consistent with the observations of 90-day repeated oral toxicity study.

INFAT® Plus was designed to comply with the Chinese National Food Safety Standard of 1, 3-Dioleoyl 2-palmitoyl triglyceride (GB 30604-2015) for infant formulas. Clinical trials conducted with sn2-palmitate have demonstrated its beneficial effects in infants on crying and sleep patterns [30,41], bone strength [31], reduced fatty acid secretion [29], microbiome [28] and other effects. Nonclinical toxicology studies have not been conducted previously with INFAT® Plus but have been conducted with a similar sn2-palmitate [42] and with palmitic acid [43]. In a 3-generation dietary reproductive toxicity study with exposures to a sn2-palmitate material ranging from 7.4 to 20.8 g/kg BW/day, there were no systemic adverse effects noted and the NOEL was considered to be the highest dose tested (20.8 g/kg BW/day for males and 16 g/kg BW/day for females). Likewise, no adverse or treatment related effects were observed in any reproductive parameters, supporting the SAMR decision to not conduct additional reproductive, neurotoxicity or immunotoxicity studies with INFAT® Plus. In the studies of palmitic acid, similar results in the acute to the current INFAT® Plus studies were observed: the LD50 was > 10 g/kg; no mutagenicity was observed in an AMES assay, and only hyperlipidemia noted in a 42-day subchronic study at 4.6 g/kg BW/day.

Other studies in laboratory animals, while not traditional toxicology studies, have shown the safety of sn2-palmitate and support the current study findings. In a 5-week dietary study in mucin deficient ($Muc2^{-/-}$) mice, animals consuming sn2-palmitate(at ~8 % of the diet) exhibited less intestinal erosion and morphological damage than control animals. The $Muc2^{-/-}$ mice normally develop spontaneous colitis due to the loss of protective abilities of the mucus layer in the intestine. There were no differences from control values in body weights or food consumption in $Muc2^{-/-}$ mice consuming the sn2-plamitate diets.[44] In a unique study on catch-up growth in feed restricted prepubertal rats, sn2-palmitate was provided to 23-day old rats for 9-days following a 17-day feed restriction (60 % of normal dietary intake). [45] The rats fed sn2-palmitate exhibited increased growth parameters (*e.g.*, bone length, epiphyseal growth plate height, bone quality) compared to the non-sn2-palmitate fed rats, while maintaining a similar body weight gain.

5. Conclusions

This study confirms that INFAT® PLUS is a nontoxic substance with LD50 values greater than 53.4 g /kg BW and 26.7 g/kg BW for ICR mice and SD rats, respectively. The result of the genotoxicity studies demonstrates that INFAT® PLUS is not genotoxic in a battery of tests using in vitro and in vivo systems. In the 90-day subchronic oral toxicity and teratogenic studies, the no-observed-adverse-effect level (NOAEL) of INFAT® PLUS was 8.9 g/kg BW under the experiment conditions. Overall, the safety data supports the use of INFAT® PLUS for infant nutrition.

Funding and data

The studies presented in this publication were sponsored, in their entirety, by IFF. Hence, IFF has the sole proprietary ownership of the results. The use of the results in this publication by stakeholders for regulatory filings, other than IFF, require a written approval by IFF.

CRediT authorship contribution statement

Arava Lavie: Conceptualization, **Zhen Liu**: Investigation, Writing – Original Draft **Jeffrey A Pitt**: Writing – Original Draft, Writing – Review & Editing, **Marina Friling**: Conceptualization, Writing – Original Draft, Writing – Review & Editing, **Song Mei**: Investigation, **Minhan Lou**:

Table 9
results of teratogenicity study for INFAT®PLUS.

Parameter	Solvent Control	INFAT® PLUS dose level		
		2.23 g/kg BW	4.45 g/kg BW	8.9 g/kg BW
Mated female				
No. of fertilized rats	19	19	19	20
No. of conceived rats	16	16	16	17
No. of corpus luteum	267	273	266	273
No. per litter	16.7 ± 2.1	17.1 ± 2.7	16.6 ± 2.9	16.1 ± 3.5
Pre-implantations mortality rate[a], n (%)	34 (12.7)	47 (17.2)	35 (13.2)	42 (15.4)
Mean rate per litter[†] (%)	0.8 ± 0.7	1.1 ± 0.9	0.8 ± 1.0	0.9 ± 0.7
Implantation rate[a], n (%)	233 (87.3)	226 (82.8)	231 (86.8)	231 (84.6)
Mean rate per litter[†] (%)	5.5 ± 0.8	5.2 ± 1.2	5.4 ± 0.9	4.9 ± 1.6
Live births rate[b], n (%)	230 (98.7)	224 (99.1)	226 (97.8)	224 (97.0)
Mean rate per litter[†] (%)	6.2 ± 1.0	6.2 ± 1.4	6.1 ± 1.1	5.7 ± 1.8
Absorbed births rate[b], n (%)	3 (1.3)	2 (0.9)	4(1.7)	5(2.2)
Mean rate per litter[†] (%)	0.08 ± 0.17	0.06 ± 0.15	0.11 ± 0.19	0.14 ± 0.33
Stillbirth rate[b], n (%)	0 (0.0)	0 (0.0)	1 (0.4)	2 (0.9)
Mean rate per litter[†] (%)	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.11	0.05 ± 0.14
Gravid uterus weight per litter (g)	85.5 ± 11.6	82.6 ± 17.3	86.9 ± 16.3	77.8 ± 23.2
Sex ratio (female:male)	1:0.95	1:1.01	1:0.93	1:1.07
Fetus growth & development				
No. of fetuses examined	230	224	226	224
Fetal weight (g)	3.79 ± 0.20	3.83 ± 0.22	3.93 ± 0.50	3.92 ± 0.25
Fetal body length (mm)	5.244 ± 0.085	5.238 ± 0.078	5.243 ± 0.235	5.196 ± 0.145
External malformations rate[c]				
Exposed brain, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Spina bifida, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Exposed belly, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cleft lip, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
No tail, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Skeletal malformation rate[d]				
No. of fetuses examined	119	116	118	118
Missing sternum, n (%)	110 (92.4)	107 (92.2)	110 (93.2)	110 (93.2)
Mean rate per litter[†] (%)	5.8 ± 1.1	5.8 ± 1.7	5.8 ± 1.5	5.5 ± 1.7
Rib deformity, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Incomplete ossification of the parietal bone, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing cervical spine, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing sacrum, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Occipital defect, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Enlarged fontanelle, n (%)	0 (0.0)	0 (0.0)	1 (0.8)	1 (0.8)
Mean rate per litter[†] (%)	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.21	0.05 ± 0.21
Visceral malformations rate[e]				
No. of fetuses examined	111	108	108	106
Nasal hyperemia, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ventricle hyperemia, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Kidney atrophy, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cleft palate, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Data expressed as mean ± standard deviation unless otherwise stated; [a]pre-implantations mortality or implantations rate (%): no. of specific event/ no. of corpus luteum x 100%; [b]live births, absorbed births or stillbirth rate (%): no. of specific event/ no. of implantations x 100%; [c] external malformations rate (%): no. of external malformations / no. of fetuses examined x 100 %; [d]missing sternum or other skeletal malformations rate (%): no. of specific event/ no. of fetuses examined for skeletal malformation x 100 %; [e]visceral malformations rate (%): no. of visceral malformations / no. of fetuses examined for visceral malformations x 100 %; Body length: height + tail length.; [†]Mean rate per litter (%) is calculated based on the overall rate (%) divided by no. of conceived rats; No significant differences were observed.

Investigation, **Xuefeng Qu**: Investigation, **Fei Hongtao**: Project administration, Writing – Review & Editing **Yin Wang**: Investigation, Supervision**, Eran Ivanir**: Conceptualization, Writing – Original Draft, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. L, J.A.P, P.R, M.F, and E.I were employees of IFF which commercializes INFAT®PLUS. Z.N, S.M, M.L, X.Q, and Y.W were part of Hangzhou Medical College, Hangzhou, China and were hired by IFF to conduct the study. F.H was part of Antion Beijing Information Consulting Co., Ltd and was hired by IFF to coordinate and manage the study.

Data Availability

The data that has been used is confidential.

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