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Twenty-eight-day oral dose toxicity evaluation of SUNACTIVE Fe-P80 in rats

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Abstract

Iron deficiency is known to be a common nutritional disorder in many countries, especially among children, women of childbearing age and pregnant women. SUNACTIVE Fe-P80 is a new type of iron supplement that applies nanotechnlateology for the purpose of overcoming the disadvantages of food supplements. This study was conducted to investigate the potential adverse effects of a 28-day repeated oral dose of SUNACTIVE Fe-P80 in rats. SUNACTIVE Fe-P80 was administered once daily by gavage to Sprague-Dawley rats for 28 days at doses of 0, 500, 1,000, and 2,000 mg/kg/day. Additional recovery groups from the control and high-dose groups were observed for a 14-day recovery period. At the scheduled termination, the animals were sacrificed, their organs weighed, and blood samples collected. There were no treatment-related effects in the context of clinical signs, body weight, food intake, ophthalmoscopy, urinalysis, necropsy findings, organ weights, and hematologic, serum biochemical and histopathological parameters at any dose tested. Under the present experimental conditions, the no-observed-adverse-effect level of SUNACTIVE Fe-P80 was ≥ 2,000 mg/kg/day in both the sexes, and no target organs were identified. Thus, the results suggest that SUNACTIVE Fe-P80 is relatively safe, as no treatment-related adverse effects were observed following a 28-day repeated oral dose experiment.

Keywords: iron; supplements; SUNACTIVE Fe-P80; subchronic toxicity; no-observed-adverse-effect level

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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INTRODUCTION

Iron is a mineral that is naturally present in many foods, is added to some food products, and is available as a dietary supplement. The characteristic of iron to stably interconvert between its most common oxidative forms, Fe^{2+} and Fe^{3+}, has been extensively exploited in biological systems [1–3]. Iron is an essential component of hemoglobin, a red blood cell protein that serves as a carrier of oxygen from the lungs to the tissues, and as an integrated part of important enzyme systems in various tissues [4]. Iron is a component of myoglobin, another oxygen-supplying protein, which regulates muscle metabolism and maintenance of connective tissue [5]. Iron is also required for physical growth, neurological development, cell function, and the synthesis of hormones such

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Ethics Approval

The Institutional Animal Care and Use Committee of Korea Testing and Research Institute approved the protocols used in this animal study, and the animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (Approved number: IAC2018-112).

as hepcidin [5, 6]. Iron intake recommendation is provided in the Dietary Reference Intakes developed by the Food and Nutrition Board (FNB) at the Institute of Medicine of the National Academy of Sciences. According to the USA Institute of Medicine [7], the Recommended Dietary allowances of iron for women and men aged 19 and above are 18 and 8 mg/day, respectively.

Iron deficiency is a common nutritional disorder in many countries, especially among young children, women of reproductive age, and pregnant women. People with iron deficiency usually have other nutrient deficiencies because iron deficiency is associated with poor diet, poor absorption, and blood loss. In developing countries, iron deficiency often results from intestinal parasites and blood loss [5]. The World Health Organization (WHO) estimates that approximately half of the 1.62 billion cases of anemia worldwide are because of iron deficiency [8]. Iron deficiency is the most common cause of anemia, but deficiencies of other micronutrients (e.g., folic acid and vitamin B12) and other factors (e.g., chronic infection and inflammation) can cause different forms of anemia or contribute to the severity of anemia. Meanwhile, excessive iron intake (more than 20 mg/kg) from supplements or medicines can lead to gastritis, constipation, nausea, stomachache, vomiting, and faintness, especially if not taken with food [5, 7]. Zinc absorption and plasma zinc concentrations can be reduced by taking iron supplements [5, 9, 10]. In severe cases, excessive iron can lead to multisystem organ failure, coma, convulsions, and even death [11, 12].

Food supplementation refers to the addition of essential trace elements and vitamins to food, to provide a public health benefits while improving nutritional quality and minimizing health risks. This is a relatively simple and efficient method to prevent mineral deficiencies. Ferrous and ferric iron salts, such as ferrous sulfate, ferrous gluconate, ferric citrate, and ferric sulfate are frequently used forms of iron in supplements [6, 12]. However, mineral fortification in foods is challenging because it often causes unexpected changes during preparation and storage, low bioavailability, and high coast [13]. Application of nanotechnology can overcome these shortcomings in food fortification, for example, by using colloidal techniques, surface coating, emulsification, etc [14]. SUNACTIVE Fe-P80 is a new type of iron supplement that applies nanotechnology for the purpose of overcoming the disadvantages of food supplements. In addition to the benefits of nanotechnology in food preservation and fortification, some researchers have highlighted significant safety concerns because it is unknown at what concentration or size nanoscopic materials may start to demonstrate novel toxicological features. The safety of nano-foods and dietary supplements must be evaluated through repeated oral dosage toxicities in *in vivo* investigations.

This study was carried out to investigate the no-observed-adverse-effect level (NOAEL) and target organs of SUNACTIVE Fe-P80. SUNACTIVE Fe-P80 was orally administered to male and female Sprague-Dawley (SD) rats for 28 days, and qualitative and quantitative tests were performed. This study was conducted in accordance with the test guidelines of the Korean Ministry of Food and Drug Safety [15] and the Organization for Economic Cooperation and Development guidelines [16] for the testing of chemicals under modern Good Laboratory Practice (GLP) regulations.

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MATERIALS AND METHODS

Experimental animals and animal husbandry

A total of 70 SD rats (35 males and 35 females) approximately 5 weeks old and within weight range of 10 g for both sexes were received from a specific pathogen-free colony at the Orient Bio (Gapyeong, Korea). Animal room temperature and humidity controls were generally within 20°C to 26°C and 40% to 70% respectively. Artificial lighting was controlled to give 12 hours continuous light and 12 hours continuous dark per 24 hours with a luminous intensity of 150–300 Lux and 10–20 air changes per hour. The rats were placed at random in suspended cages with wire mesh floors with stainless steel wire cage (W310 × L500 × H200 mm), according to sex, so that each cage contained 5 rats of the same sex. After an acclimatization period of 7 days, each animal was weighed and the required number of animals was selected by discarding those animals furthest from the mean bodyweight. The remaining animals were then randomly assigned to cages, stratified by bodyweight, in such a way that the initial cage means were approximately equal. The appropriate numbers of cages were then allocated to each treatment group. At the commencement of the study, the range of bodyweight variation in the animals used did not exceed 20% of the appropriate mean values. Later, the group size was decreased to two animals per cage during the administration and observation periods. The proper laboratory animal pellet meal (Labdiet 5L79, PMI Nutrition International, St. Louis, MO, USA), which was acquired from Orient Bio, was made available to the animals at all times. Underground water was provided freely in the form of a water bottle after being sterilized with UV light and put through ultrafiltration. The protocols utilized in this animal study were approved by Korea Testing and Research Institute's Institutional Animal Care and Use Committee, and the animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (Approved number: IAC2018-112) [17].

Preparation of test substance

Before usage, SUNACTIVE Fe-P80 (26.6% ferric pyrophosphate, 54.7% dextrin, 7.3% sodium chloride, 5.3% glycerol esters of fatty acids, 4.8% water, and 1.3% enzymatically hydrolyzed lecithin) was kept at room temperature (15°C to 25°C) which was provided by Taiyo Kagaku (Yokkaichi, Japan). Orally administered sterile distilled water (Daihan Pharm, Seoul, Korea), which was stored under refrigeration (0°C–10°C) after opening, was used as the vehicle control. SUNACTIVE Fe-P80 for oral administration was prepared by suspending it in sterile distilled water at the appropriate dose for each assigned group. SUNACTIVE Fe-P80 was analyzed by XRD (Bruker AXS D2 Phaser, Bruker, Billerica, MA, USA) to obtain an amorphous phase. It was then measured using a dynamic light scattering instrument (ELSZ-1000, Otsuka, Tokyo, Japan) to obtain 240 nm hydrodynamic radii. It showed a sphere-like morphology at low magnification, which seems to be attributed to the organic moiety, such as dextrin and glycerides. Primary particle size was shown in magnified scanning electron microscopy (Quanta 250 FEG, FEI Company, Hilsboro, OR, USA) pictures to be 40 ± 10 nm.

During the administration period, the concentration and/or homogeneity of SUNACTIVE

Fe-P80 at 50, 100, and 200 mg/mL dosages in sterile distilled water were examined by ICP-AES (HORIBA JOBIN YVON ULTIMA2, HORIBA, Longjumeau, France) and found to be within the permitted limits (concentration nominal value ± 10%; homogeneity within 5%).

Dosage selection and experimental groups

This study was conducted according to OECD testing guidelines 407 [16]. The dose levels were selected based on the results from a previous 7-day study previously performed in the SD rat where administration up to the highest dose of 2,000 mg/kg/day resulted in no mortalities or clinical signs, no treatment effect on body weight or food consumption, no effect on organ weight and no macroscopic findings in either sex (data not shown). Based on the results of a preliminary study, a high dose of 2,000 mg/kg/day was administered. Doses of 1,000 and 500 mg/kg/day were set as the middle and low doses, respectively, using a common ratio of 2. The vehicle control group was orally administered sterile water only. Each group consisted of 5 (low and middle dose groups) or 10 (vehicle control and high-dose groups) rats of each sex. In order to monitor the reversibility, persistence, or delayed development of toxic effects for 14 days after treatment, there were recovery groups of five animals per sex in both the high-dose and control dose groups.

Administration

The oral method was chosen because that is how supplements are typically given to people. Using a sonde and syringe, the prepared test material was immediately injected into the stomach. The most current body weight data were utilized to determine the dosing volume, which was 10 mL/kg/day. For 28 days, the test material was given every day.

Clinical signs, body weight, and food consumption

Animals were checked for moribundity and mortality twice daily. Animals were observed for clinical signs at least once daily for all animals during the study. Detailed physical examinations were performed at least weekly during the treatment and recovery periods. The nature, onset, severity, duration and recovery of clinical signs were recorded. Cages and cage trays were inspected daily for evidence of ill health such as blood or loose feces. Each animal was weighed three times during the acclimatization period, on the first day of test item administration, then at least weekly throughout the treatment and recovery periods. Additionally, diet fasted animals were weighed before scheduled necropsy (terminal body weight). The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment and recovery periods. From these records the mean daily consumption was calculated.

Ophthalmoscopy

During the acclimatization period, all animals were subjected to an ophthalmic examination. After instillation of an atropinic agent (Mydriatics, Ocuhomapin, Samil Pharm, Seoul, Korea) each eye was examined by means of an indirect ophthalmoscope (Genesis, Kowa, Nagoya, Ja-

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pan). The anterior eye structures were examined, as well as the eye when the pupil had dilated. During day 28 of the treatment period, all surviving animals from control and high dose group were re-examined.

Urinalysis

On study Day 27 (for animals of the main study phase) and on recovery Day 13 (for animals of the recovery phase), in the morning, overnight urine samples were collected from all surviving animals in all groups. Five animals from each group were sampled on each day. Food and water were not accessible during urine collection. Urine samples were weighed to determine urinary volume.

The bilirubin, glucose, specific gravity, ketone bodies, pH, occult blood, protein, nitrite, urobilinogen, and white blood cell count were assayed using an automatic analyzer (Clini-Tek 500, Ames Division, Miles Laboratory, Elkhart, IN, USA).

Urinary refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France). Microscopic examination of the urinary sediment was performed after centrifugation (425 RCF, MF300, Hanil, Gimpo, Korea) of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

Hematology

On the day of scheduled sacrifice (Study Day 28 for animals of the main study and on Recovery Day 14 for animals of the recovery phase), blood samples were taken from the vena cava of all surviving animals in all groups. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane (Virbac, Carros, France). Blood was collected on EDTA for hematology (approximately 0.5 mL) and on sodium citrate for coagulation parameters (approximately 0.9 mL). Hematology parameters were measured using an automatic hematology analyzer Coulter counter (ADVIA 120E, Siemens, PA, USA): WBC, WBC differential counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), reticulocytes, hematocrit, mean corpuscular hemoglobin (MCH) concentration, hemoglobin concentration, mean corpuscular volume, platelet count, red blood cell count, and MCH. In order to measure the time it takes for blood to clot, plasma was taken and centrifuged (735 RCF, Microcentrifuges 5402, Eppendorf, Hamburg, Germany) for 10 min. The nephelometric analysis method was used to measure the prothrombin time and activated partial thromboplastin time in seconds using a coagulation time analyzer (ACL 7000, Instrumentation Laboratory, Bedford, MA, USA).

Serum biochemistry

For serum clinical chemistry, blood was drawn through a clot activator (about 1.1 mL) and let to sit at room temperature for 90 minutes. Then, it was centrifuged (5,000 × g, 10 min), and the serum was separated for hematological analysis. Parameters were measured using a serum biochemistry analyzer (TBA-120FR, TOSHIBA, Tokyo, Japan): alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bile acid, total bilirubin, creatine phos-

phokinase, glucose, triglyceride, total cholesterol, albumin, albumin/globulin ratio, total protein, creatinine, blood urea nitrogen, gamma-glutamyl transpeptidase, inorganic phosphorus, ferritin, sodium ion, chloride ions, potassium ion, and calcium.

Necropsy

On study Days 28 of the dosing phase for the 28-day treatment phase, on Days 14 of the recovery phase for the reversibility sacrifice, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. All significant organs, tissues, and body cavities were examined during the necropsy. Macroscopic abnormalities were recorded, sampled and examined microscopically.

Organ weights

The brain, heart, kidneys, spleen, epididymides, testes, prostate gland (prostate and seminal vesicles with coagulating glands), ovaries, adrenal glands, thymus, uterus, and liver were weighed at necropsy for all animals scheduled for euthanasia. Organ pairs were weighed independently. Based on the terminal body weight, relative organ weight was determined.

Histopathology

All of the major organs and tissues of each animal were macroscopically inspected after a thorough necropsy. Histopathological evaluation of all organs and tissues from the control and high-dose groups was performed. The heart, thymus, brain, lungs, spleen, kidneys, aorta, trachea, nasal cavity, liver, pituitary, parathyroid glands, thyroid, adrenal glands, tongue, stomach, esophagus, salivary glands, jejunum, cecum, duodenum, colon, ileum, rectum, urinary bladder, uterus, pancreas, ovaries, seminal vesicles, mammary glands, vagina, epididymides, testes, prostate, skin, cervical and mesenteric lymph nodes, bulbourethral glands, trigeminal nerve, sternum and femur including bone, marrow, spinal cord (cervical, thoracic, and lumbar cord), sciatic nerve, Harderian glands, eyes, thigh muscle, and gross abnormalities were excised.

Except for the testicles, which were fixed in Bouin solution, and the eyes, including the optic nerve and Harderian glands, which were fixed in Davidson's fixative, all of the aforementioned organs and tissues were fixed in 10% buffered formalin. All of the above samples were embedded in paraffin wax, as previously described [18, 19]. Histological sections, stained with hematoxylin and eosin, were prepared from all organs and tissue samples from all the animals in the control and high dose groups and from animals which died or were sacrificed before scheduled sacrifice/from all animals. Specimens were then processed for histopathology at the Preclinical Research Center, Korea Testing & Research Institute (Hwasun, Korea), which is a GLP institute approved by the MFDS.

Statistical analysis

Male and female statistical analyses were carried out separately. Numerical data are pre-

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sented as means with S.D. for each group. Food consumption, body weight, hematology, urine volume, organ weights and serum biochemistry were assumed to be normally distributed and analyzed by one-way analysis of variance and Student's *t*-test for recovery group comparisons. Using Levene's test, the homogeneity presumption was examined. If the assumption of homogeneity of variance was not met, Dunnett's T3 test was used as the post-hoc test [20]. Urinalysis data were rank-transformed and analyzed using the non-parametric Kruskal-Wallis H-test [21]. If a statistically significant difference was observed between the groups, the Mann-Whitney U-test was used to identify the groups that were significantly different from the vehicle control group [22].

For categorical data, including estrous cycle, clinical signs, necropsy, and histopathological findings, the proportion of animals was analyzed using Fisher's exact probability test [23] for each treated group versus the control group. SPSS version 19.0 was used for all statistical analyses (SPSS, Chicago, IL, USA). The significance of the differences between the control and treatment groups was estimated at probability levels of 1% and 5%.

RESULTS

Clinical signs, body weight, and food consumption

Throughout the study period, there were no treatment-related clinical signs, deaths, or moribund sacrifices (data not shown). As shown in Table 1, there were no significant differences in body weight between the vehicle control and the treatment groups during the experimental period in either sex. Changes in food consumption in SUNACTIVE Fe-P80-treated rats are summarized in Table 2. Results showed that food consumption significantly decreased on day 8 of the test in the male 500 and 1,000 mg/kg/day groups compared to the vehicle control group ($p<0.01$). In females, however, no significant differences were observed between the vehicle control and treatment groups.

Table 1. Mean body weight changes of rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Items	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of males examined	5	5	5	5
Day 0	177.1 ± 3.1	176.7 ± 6.4	173.7 ± 1.9	176.4 ± 7.2
Day 7	231.3 ± 10.5	230.4 ± 13.5	224.3 ± 9.1	238.2 ± 10.5
Day 14	280.4 ± 14.5	281.8 ± 22.5	271.3 ± 16.7	297.7 ± 16.5
Day 21	314.2 ± 17.5	318.8 ± 28.8	305.2 ± 21.7	339.1 ± 23.7
Day 27	339.6 ± 19.5	350.6 ± 33.9	328.2 ± 26.8	366.3 ± 32.8
No. of females examined	5	5	5	5
Day 0	153.6 ± 6.5	151.7 ± 5.8	152.4 ± 4.3	154.6 ± 2.3
Day 7	177.6 ± 6.9	178.7 ± 11.7	180.0 ± 10.9	178.3 ± 6.0
Day 14	204.2 ± 6.4	198.6 ± 17.3	203.1 ± 19.9	197.8 ± 10.6
Day 21	224.3 ± 8.9	214.9 ± 20.6	227.1 ± 18.7	215.9 ± 8.1
Day 27	239.2 ± 7.3	229.9 ± 22.0	243.5 ± 20.9	228.9 ± 7.4

Values are presented as means ± S.D. (g).

Table 2. Mean food consumption of rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Items	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of males examined	5	5	5	5
Day 1	29.5 ± 1.1	28.2 ± 2.2	27.7 ± 2.8	30.4 ± 1.4
Day 8	33.3 ± 0.8	29.7 ± 1.8**	29.1 ± 0.2**	32.1 ± 1.8
Day 15	32.8 ± 1.0	31.0 ± 1.5	29.6 ± 3.0	34.8 ± 2.5
Day 22	29.6 ± 1.4	29.9 ± 1.1	28.1 ± 2.5	30.6 ± 1.6
No. of females examined	5	5	5	5
Day 1	19.7 ± 1.6	19.4 ± 5.1	19.9 ± 3.8	22.5 ± 2.2
Day 8	23.0 ± 0.8	20.6 ± 5.3	20.0 ± 5.5	21.6 ± 3.8
Day 15	25.6 ± 2.0	21.7 ± 6.0	21.5 ± 3.2	22.2 ± 2.0
Day 22	23.6 ± 2.1	19.3 ± 5.1	20.4 ± 5.7	20.6 ± 2.1

Values are presented as means ± S.D. (g).

** Significantly different from the vehicle control group at $p<0.01$.

Urinalysis and ophthalmic examination

No treatment-related changes were observed in urinalysis- and ophthalmic examination-related parameters at the end of the experimental period (data not shown).

Hematology

Results of the hematological examination are shown in Tables 3 and 4. MCH for males in the 2,000 mg/kg/day group was significantly increased ($p<0.05$) compared with that for the males in the vehicle control group. Neutrophil count was significantly increased ($p<0.05$) in the female 1,000 mg/kg/day group, compared to the vehicle control group. Significant changes in the hematological parameters observed in the males and females of the treatment groups were not observed at the end of the recovery period (data not shown).

Serum biochemistry

Serum biochemistry results are shown in Tables 5 and 6. Triglyceride levels in males in the 1,000 mg/kg/day group were significantly decreased ($p<0.05$) compared with those in males in the vehicle control group. In females, however, no significant differences were observed between the vehicle control and treatment groups. At the end of the recovery period, no significant differences in serum biochemical parameters were observed between the vehicle control and highest dose groups (data not shown).

Gross findings and organ weights

At the planned necropsy, no treated animals displayed any gross findings attributable to the treatment (data not shown). The absolute and relative organ weights of the SUNACTIVE Fe-P80-treated male and female rats are presented in Tables 7 and 8. No treatment-related changes were observed in absolute or relative organ weights at the end of the experimental period between the groups.

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Table 3. Hematological values of male rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Parameter	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of males examined	5	5	5	5
RBC (10^6/μL)	7.8 ± 0.4	7.6 ± 0.4	7.9 ± 0.5	7.4 ± 0.2
Hemoglobin (g/dL)	15.4 ± 0.7	15.4 ± 0.5	15.9 ± 0.8	15.3 ± 0.4
Hematocrit (%)	43.4 ± 2.0	43.7 ± 1.1	45.6 ± 1.4	42.7 ± 1.2
MCV (fL)	55.4 ± 1.2	57.7 ± 2.2	57.7 ± 2.1	57.9 ± 2.8
MCH (pg)	19.6 ± 0.3	20.3 ± 0.6	20.1 ± 0.5	20.8 ± 0.7*
MCHC (g/dL)	35.4 ± 0.4	35.2 ± 0.6	34.9 ± 0.7	35.9 ± 0.7
Reticulocyte (10^9/L)	207.2 ± 26.7	227.9 ± 42.4	218.3 ± 58.3	250.0 ± 72.5
Reticulocyte (%)	2.7 ± 0.4	3.0 ± 0.6	2.8 ± 0.9	3.4 ± 0.8
Platelet (10^3/μL)	1,108.0 ± 152.2	1,085.2 ± 103.6	1,073.8 ± 164.0	1,124.8 ± 146.1
WBC (10^3/μL)	5.70 ± 1.16	5.40 ± 1.17	5.64 ± 1.78	6.07 ± 2.50
Neutrophils (10^3/μL)	0.83 ± 0.15	0.78 ± 0.20	1.00 ± 0.48	0.56 ± 0.29
Neutrophils (%)	15.14 ± 4.50	14.80 ± 3.94	18.90 ± 8.49	11.14 ± 7.80
Lymphocytes (10^3/μL)	4.62 ± 1.08	4.35 ± 1.08	4.41 ± 1.71	5.21 ± 2.29
Lymphocytes (%)	80.52 ± 4.16	80.22 ± 4.98	77.16 ± 8.98	84.00 ± 7.57
Monocytes (10^3/μL)	0.13 ± 0.07	0.15 ± 0.03	0.12 ± 0.07	0.16 ± 0.07
Monocytes (%)	2.26 ± 1.10	2.84 ± 0.64	2.06 ± 0.73	2.58 ± 0.37
Eosinophils (10^3/μL)	0.07 ± 0.01	0.07 ± 0.03	0.07 ± 0.03	0.06 ± 0.02
Eosinophils (%)	1.26 ± 0.24	1.28 ± 0.59	1.26 ± 0.36	1.10 ± 0.34
Basophils (10^3/μL)	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Basophils (%)	0.16 ± 0.05	0.16 ± 0.09	0.12 ± 0.08	0.22 ± 0.13
PT (sec)	13.8 ± 0.5	13.7 ± 0.7	13.9 ± 0.8	13.3 ± 0.7
APTT (sec)	21.8 ± 1.2	19.9 ± 2.7	20.8 ± 1.7	19.9 ± 2.1

Values are presented as means ± S.D.

* Significantly different from the vehicle control group at $p<0.05$.

RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell count; PT, prothrombin time; APTT, active partial thromboplastin time.

Histopathology

The results of the histopathological examination are presented in Table 9 and Fig. 1. Inflammatory cell infiltration in the liver was observed in one and two males in the vehicle control and 2,000 mg/kg/day groups, respectively, and focal necrosis in the liver was observed in one male in the vehicle control group. Interstitial inflammatory cell infiltration in the kidney was observed in one male in the 2,000 mg/kg/day group. Tubular dilation in the kidney was observed in one female in the 2,000 mg/kg/day group, and tubular cast in the kidney was observed in one female in the vehicle control group. Vacuolation in the pituitary glands was observed in one male in the 2,000 mg/kg/day group. Extramedullary hematopoiesis in the spleen was observed in one male each in the vehicle control and 2,000 mg/kg/day groups. Interstitial inflammatory cell infiltration in the pancreas was observed in one male in the 2,000 mg/kg/day group. At the end of the recovery period, no histopathological alterations in the liver, kidney, pituitary glands, spleen, or pancreas were observed in the highest dose group in either sex (data not shown).

Table 4. Hematological values of female rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Parameter	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of females examined	5	5	5	5
RBC (10^6/μL)	7.4 ± 0.3	7.3 ± 0.4	7.3 ± 0.3	7.1 ± 0.2
Hemoglobin (g/dL)	15.0 ± 0.6	15.1 ± 1.0	15.0 ± 0.4	14.6 ± 0.1
Hematocrit (%)	41.1 ± 1.6	41.4 ± 2.8	41.0 ± 1.5	39.5 ± 1.0
MCV (fL)	55.5 ± 1.6	56.8 ± 2.4	56.1 ± 1.4	55.6 ± 1.2
MCH (pg)	20.2 ± 0.6	20.7 ± 0.7	20.6 ± 0.6	20.5 ± 0.6
MCHC (g/dL)	36.5 ± 0.3	36.4 ± 0.7	36.6 ± 0.5	36.9 ± 0.8
Reticulocyte (10^9/L)	186.3 ± 35.3	193.5 ± 56.1	223.9 ± 20.0	249.9 ± 109.3
Reticulocyte (%)	2.5 ± 0.5	2.7 ± 0.9	3.1 ± 0.2	3.5 ± 1.6
Platelet (10^3/μL)	1,195.6 ± 95.5	1,106.4 ± 93.4	1,230.2 ± 151.7	1,299.8 ± 146.3
WBC (10^3/μL)	5.31 ± 1.03	4.73 ± 1.26	5.46 ± 0.96	5.05 ± 0.75
Neutrophils (10^3/μL)	0.61 ± 0.06	0.75 ± 0.32	0.77 ± 0.06*	0.62 ± 0.17
Neutrophils (%)	11.72 ± 1.96	16.20 ± 6.22	14.44 ± 2.45	12.30 ± 2.76
Lymphocytes (10^3/μL)	4.42 ± 0.94	3.76 ± 1.09	4.40 ± 0.87	4.19 ± 0.72
Lymphocytes (%)	83.16 ± 2.00	79.20 ± 6.33	80.38 ± 2.27	82.72 ± 2.72
Monocytes (10^3/μL)	0.13 ± 0.07	0.09 ± 0.03	0.15 ± 0.06	0.11 ± 0.05
Monocytes (%)	2.36 ± 0.75	1.90 ± 0.34	2.68 ± 0.64	2.34 ± 1.24
Eosinophils (10^3/μL)	0.10 ± 0.03	0.08 ± 0.03	0.09 ± 0.04	0.09 ± 0.02
Eosinophils (%)	1.84 ± 0.63	1.76 ± 0.18	1.72 ± 0.76	1.78 ± 0.52
Basophils (10^3/μL)	0.01 ± 0.00	0.00 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Basophils (%)	0.14 ± 0.05	0.12 ± 0.04	0.16 ± 0.05	0.14 ± 0.05
PT (sec)	13.4 ± 0.6	13.6 ± 0.4	13.3 ± 0.6	13.3 ± 0.2
APTT (sec)	17.7 ± 1.4	17.7 ± 1.1	18.3 ± 1.6	18.8 ± 1.0

Values are presented as means ± S.D.

* Significantly different from the vehicle control group at $p<0.05$.

RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell count; PT, prothrombin time; APTT, active partial thromboplastin time.

DISCUSSION

In order to establish the NOAEL and target organs, this study examined the possible subchronic toxicity of SUNACTIVE Fe-P80 in rats after a 28-day repeated oral treatment. The findings of this investigation demonstrated that repeated oral administration of SUNACTIVE Fe-P80 to rats for 28 days at doses of less than 2,000 mg/kg/day did not result in any treatment-related side effects.

Our results showed that 28-day repeated oral administration of SUNACTIVE Fe-P80 did not result in any clinical signs of toxicity or death at any dose tested in either sex. No abnormal changes in body weight or food consumption were observed during the treatment or recovery period. The significant decreases in food consumption observed in the male 500 and 1,000 mg/kg/day groups were not considered to be toxicologically significant because they were not dose-related, and were unaccompanied by correlated changes in other parameters.

The significant changes in hematological and serum biochemical parameters (MCH,

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Table 5. Serum biochemical values of male rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Parameter	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of males examined	5	5	5	5
AST (IU/L)	124.2 ± 42.3	111.4 ± 30.3	96.0 ± 22.2	115.6 ± 39.0
ALT (IU/L)	30.6 ± 5.1	31.8 ± 4.7	29.4 ± 2.5	30.2 ± 5.4
ALP (IU/L)	555.6 ± 69.1	642.6 ± 130.0	576.8 ± 72.9	508.6 ± 82.0
CPK (IU/L)	883.6 ± 599.6	644.8 ± 422.8	464.8 ± 286.7	739.2 ± 463.7
TBIL (mg/dL)	0.020 ± 0.015	0.016 ± 0.004	0.019 ± 0.012	0.022 ± 0.049
TBA (μmol/L)	12.6 ± 3.5	15.4 ± 5.0	14.6 ± 11.4	14.1 ± 5.9
Glucose (mg/dL)	144.2 ± 31.4	150.2 ± 48.5	147.2 ± 23.5	150.8 ± 21.2
TCHO (mg/dL)	67.6 ± 9.5	54.4 ± 11.3	59.2 ± 24.7	62.4 ± 7.7
Triglyceride (mg/dL)	38.4 ± 9.3	25.6 ± 4.5	17.0 ± 6.7*	30.4 ± 9.3
TP (g/dL)	5.6 ± 0.2	5.8 ± 0.1	6.0 ± 0.4	5.9 ± 0.1
Albumin (g/dL)	3.6 ± 0.1	3.7 ± 0.0	3.7 ± 0.2	3.7 ± 0.1
A/G (ratio)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
BUN (mg/dL)	13.0 ± 1.2	13.5 ± 2.4	13.9 ± 3.0	13.8 ± 1.5
Creatinine (mg/dL)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0
IP (mg/dL)	8.2 ± 0.6	9.1 ± 1.7	9.1 ± 2.4	8.2 ± 0.5
GGT (IU/L)	0.4 ± 0.2	0.7 ± 1.1	0.5 ± 0.4	0.3 ± 0.2
Ca^{++} (mg/dL)	8.7 ± 0.8	9.4 ± 0.5	9.4 ± 0.5	9.7 ± 0.3
Na^{+} (mmol/L)	145.3 ± 0.4	145.2 ± 1.4	145.5 ± 0.4	144.6 ± 1.2
K^{+} (mmol/L)	4.8 ± 0.4	4.9 ± 0.4	4.9 ± 1.1	4.9 ± 0.4
Cl^{-} (mmol/L)	106.1 ± 1.0	105.7 ± 1.6	107.2 ± 2.5	105.4 ± 1.5
Fe-L (μg/dL)	98.6 ± 11.7	131.2 ± 100.5	93.5 ± 9.6	95.3 ± 15.5

Values are presented as means ± S.D.

* Significantly different from the vehicle control group at $p<0.05$.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; TBIL, total bilirubin; TBA, total bile acid; TCHO, total cholesterol; TP, total protein; A/G, albumin/globulin; BUN, blood urea nitrogen; IP, inorganic phosphorus; GGT, gamma glutamyl transpeptidase; Fe-L, ferritin.

neutrophils, and triglycerides) observed in the treatment groups were also considered incidental since the findings lacked a dose-response relationship and were not accompanied by other relevant findings. Moreover, such changes were within an acceptable historical range [24, 25].

No abnormal changes in necropsy findings or absolute and relative organ weights were observed at any dose tested. Although some histopathological alterations, including inflammatory cell infiltration in the liver, interstitial inflammatory cell infiltration, and tubular dilation in the kidney, vacuolation in the pituitary glands, extramedullary hematopoiesis in the spleen, and interstitial inflammatory cell infiltration in the pancreas, were observed in the 2,000 mg/kg/day group for both sexes, they were considered to be incidental findings, as their incidence and severity were similar to those in their respective control groups. Moreover, these findings are commonly observed in normal control rats [19, 26–28].

Serum iron and ferritin are the most commonly used hematological measures to assess the effect of iron supplementation. Unlike serum iron, serum ferritin concentration does not fluctuate from day to day. Because of its relatively high stability and solubility, as well as

Table 6. Serum biochemical values of female rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Parameter	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of females examined	5	5	5	5
AST (IU/L)	114.8 ± 6.4	119.0 ± 36.2	125.0 ± 24.2	104.2 ± 20.0
ALT (IU/L)	28.8 ± 4.1	34.2 ± 13.0	29.0 ± 7.4	26.4 ± 4.4
ALP (IU/L)	355.2 ± 45.6	456.6 ± 85.6	351.6 ± 87.2	337.6 ± 47.9
CPK (IU/L)	696.6 ± 163.0	605.0 ± 295.7	966.2 ± 391.5	654.8 ± 337.4
TBIL (mg/dL)	0.014 ± 0.009	0.018 ± 0.015	0.006 ± 0.009	0.020 ± 0.035
TBA (μmol/L)	14.7 ± 6.5	18.0 ± 15.0	12.9 ± 5.4	14.9 ± 3.5
Glucose (mg/dL)	134.0 ± 23.6	134.0 ± 16.8	141.6 ± 13.0	138.8 ± 19.7
TCHO (mg/dL)	65.6 ± 3.6	70.0 ± 9.7	81.0 ± 20.7	81.0 ± 11.4
Triglyceride (mg/dL)	9.6 ± 1.7	13.4 ± 5.5	12.2 ± 2.0	12.8 ± 7.9
TP (g/dL)	6.0 ± 0.1	5.8 ± 0.2	5.9 ± 0.2	6.1 ± 0.5
Albumin (g/dL)	3.8 ± 0.1	3.7 ± 0.2	3.8 ± 0.1	4.0 ± 0.4
A/G (ratio)	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.0	1.9 ± 0.2
BUN (mg/dL)	16.6 ± 2.4	18.9 ± 3.4	17.9 ± 2.4	15.9 ± 2.0
Creatinine (mg/dL)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.0
IP (mg/dL)	7.4 ± 0.9	7.8 ± 1.0	7.2 ± 0.9	6.0 ± 1.0
GGT (IU/L)	0.3 ± 0.4	0.2 ± 0.4	0.0 ± 0.1	0.1 ± 0.1
Ca^{++} (mg/dL)	9.3 ± 0.1	9.3 ± 0.2	9.4 ± 0.3	9.3 ± 0.3
Na^{+} (mmol/L)	142.8 ± 1.1	144.3 ± 1.1	143.4 ± 0.9	143.7 ± 2.2
K^{+} (mmol/L)	4.3 ± 0.3	4.2 ± 0.0	4.2 ± 0.3	4.2 ± 0.3
Cl^{-} (mmol/L)	106.1 ± 1.4	107.3 ± 1.4	105.3 ± 0.7	107.7 ± 1.6
Fe-L (μg/dL)	202.3 ± 60.2	225.3 ± 55.3	193.2 ± 95.1	188.8 ± 48.5

Values are presented as means ± S.D.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; TBIL, total bilirubin; TBA, total bile acid; TCHO, total cholesterol; TP, total protein; A/G, albumin/globulin; BUN, blood urea nitrogen; IP, inorganic phosphorus; GGT, gamma glutamyl transpeptidase; Fe-L, ferritin.

its direct proportionality to body iron stores in normal persons, serum ferritin is the most commonly used indicator of total body iron storage. In this study, serum ferritin levels did not increase with a dose-response relationship. However, the animals were not in iron deficiency, and iron absorbed by gavage is homeostatic in the body, and the rest is send out of the body. So, SUNACTIVE Fe-P80 can be expected to be safe even when exposed due to increased capacity.

To date, the toxicity of many micronutrients such as iron is well understood, but the data available on the concentration or size will begin to exhibit new toxicological properties because the nanoscopic dimensions are limited. Previous studies have shown that nanoparticles may cause several cytotoxic and genotoxic effects owing to their unique properties, such as a large surface area-to-mass ratio [29, 30]. Nanoparticles are internalized by cells, resulting in genetic mutations through interactions with cellular organelles. The causes of genetic toxicity induced by nanomaterials are either direct interactions with genetic materials (DNA and RNA) or indirect damage by reactive oxygen species (ROS) [31, 32]. ROS generation by nanomaterials has been observed both *in vitro* and *in vivo* [33–37]. Recently, it was also reported that TiO2 nanoparticles can induce tumor-like changes human

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Table 7. Absolute and relative organ weights of male rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Parameter	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of males examined	5	5	5	5
Body weight (g)	313.6 ± 18.8	325.9 ± 31.8	333.9 ± 39.4	341.0 ± 33.6
Liver (g)	9.04 ± 0.85	9.81 ± 1.32	8.71 ± 0.97	10.33 ± 1.53
Per body weight (%)	2.88 ± 0.10	3.00 ± 0.14	2.66 ± 0.54	3.02 ± 0.16
Kidney-left (g)	1.26 ± 0.09	1.30 ± 0.13	1.19 ± 0.14	1.22 ± 0.11
Per body weight (%)	0.40 ± 0.01	0.40 ± 0.03	0.36 ± 0.07	0.36 ± 0.03
Kidney-right (g)	1.28 ± 0.10	1.35 ± 0.14	1.20 ± 0.11	1.10 ± 0.55
Per body weight (%)	0.41 ± 0.01	0.42 ± 0.03	0.37 ± 0.06	0.31 ± 0.16
Heart (g)	1.30 ± 0.11	1.22 ± 0.13	1.25 ± 0.15	1.30 ± 0.06
Per body weight (%)	0.41 ± 0.02	0.37 ± 0.02	0.38 ± 0.07	0.38 ± 0.04
Brain (g)	2.05 ± 0.10	2.12 ± 0.06	2.04 ± 0.11	2.08 ± 0.03
Per body weight (%)	0.65 ± 0.04	0.66 ± 0.06	0.62 ± 0.09	0.61 ± 0.06
Spleen (g)	0.66 ± 0.11	0.66 ± 0.12	0.63 ± 0.08	0.77 ± 0.10
Per body weight (%)	0.21 ± 0.03	0.20 ± 0.03	0.19 ± 0.04	0.23 ± 0.03
Thymus (g)	0.47 ± 0.04	0.54 ± 0.05	0.50 ± 0.09	0.55 ± 0.08
Per body weight (%)	0.15 ± 0.02	0.17 ± 0.03	0.15 ± 0.04	0.16 ± 0.01
Adrenal gland-left (g)	0.038 ± 0.005	0.040 ± 0.006	0.035 ± 0.008	0.036 ± 0.006
Per body weight (%)	0.012 ± 0.001	0.012 ± 0.002	0.011 ± 0.003	0.011 ± 0.003
Adrenal gland-right (g)	0.038 ± 0.006	0.039 ± 0.007	0.032 ± 0.007	0.032 ± 0.002
Per body weight (%)	0.012 ± 0.001	0.012 ± 0.002	0.010 ± 0.002	0.010 ± 0.001
Testis-left (g)	1.57 ± 0.17	1.55 ± 0.09	1.60 ± 0.17	1.56 ± 0.04
Per body weight (%)	0.50 ± 0.05	0.48 ± 0.05	0.48 ± 0.05	0.46 ± 0.04
Testis-right (g)	1.59 ± 0.22	1.57 ± 0.12	1.57 ± 0.17	1.58 ± 0.09
Per body weight (%)	0.51 ± 0.06	0.48 ± 0.04	0.47 ± 0.05	0.47 ± 0.04
Epididymis-left (g)	0.56 ± 0.03	0.52 ± 0.04	0.53 ± 0.04	0.53 ± 0.05
Per body weight (%)	0.18 ± 0.01	0.16 ± 0.02	0.16 ± 0.02	0.16 ± 0.02
Epididymis-right (g)	0.55 ± 0.03	0.54 ± 0.03	0.54 ± 0.07	0.56 ± 0.04
Per body weight (%)	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.02	0.16 ± 0.02
Prostate gland (g)	2.62 ± 0.29	2.46 ± 0.18	2.36 ± 0.28	2.63 ± 0.31
Per body weight (%)	0.84 ± 0.11	0.76 ± 0.11	0.71 ± 0.09	0.78 ± 0.14

Values are presented as means ± S.D.

cells exposed to them [38–40]. In contrast, it was found that toxicity may be effectively prevented through surface chemical changes of nanoparticles [41–44]. The surface chemistry and surface charge of nanoparticles play important roles in toxicity and corresponding safety assessments [45]. For example, polysaccharide coatings have been used to promote biocompatibility and better dispersion of solutions [46, 47]. With regard to the surface charge, positively charged Au nanoparticles caused greater toxicity than negatively charged particles [48]. Further, Al_2O_3 nanoparticles were less toxic than metallic Al nanoparticles, suggesting that surface oxide formation may also change bio-interaction [49]. In addition, ultra-small (~4–5 nm) nanodiamonds, various functionalized carbon nanotubes, and cerium nanoparticles have not shown any distinct toxic effects on cells in culture [50–55]. The results of this study

Table 8. Absolute and relative organ weights of female rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Parameter	Dose (mg/kg/day)			
	0	500	1,000	2,000
No. of females examined	5	5	5	5
Body weight (g)	218.8 ± 7.7	212.9 ± 22.2	223.7 ± 19.8	211.7 ± 5.7
Liver (g)	6.41 ± 0.42	6.64 ± 0.92	7.14 ± 1.09	6.37 ± 0.72
Per body weight (%)	2.93 ± 0.18	3.11 ± 0.19	3.18 ± 0.23	3.01 ± 0.30
Kidney-left (g)	0.87 ± 0.06	0.93 ± 0.11	0.99 ± 0.14	0.87 ± 0.08
Per body weight (%)	0.40 ± 0.02	0.44 ± 0.05	0.44 ± 0.03	0.41 ± 0.03
Kidney-right (g)	0.89 ± 0.08	0.95 ± 0.10	0.79 ± 0.44	0.87 ± 0.08
Per body weight (%)	0.41 ± 0.03	0.44 ± 0.03	0.35 ± 0.19	0.41 ± 0.03
Heart (g)	0.92 ± 0.07	0.93 ± 0.13	0.96 ± 0.09	0.96 ± 0.18
Per body weight (%)	0.42 ± 0.03	0.44 ± 0.05	0.43 ± 0.01	0.45 ± 0.08
Brain (g)	1.96 ± 0.09	1.97 ± 0.10	2.02 ± 0.10	2.04 ± 0.06
Per body weight (%)	0.90 ± 0.03	0.93 ± 0.09	0.91 ± 0.09	0.96 ± 0.03
Spleen (g)	0.57 ± 0.08	0.55 ± 0.08	0.61 ± 0.11	0.59 ± 0.09
Per body weight (%)	0.26 ± 0.03	0.26 ± 0.03	0.27 ± 0.03	0.28 ± 0.03
Thymus (g)	0.55 ± 0.09	0.56 ± 0.15	0.55 ± 0.10	0.53 ± 0.13
Per body weight (%)	0.25 ± 0.04	0.26 ± 0.05	0.24 ± 0.03	0.25 ± 0.06
Adrenal gland-left (g)	0.044 ± 0.006	0.045 ± 0.010	0.049 ± 0.006	0.040 ± 0.005
Per body weight (%)	0.020 ± 0.003	0.021 ± 0.002	0.022 ± 0.003	0.019 ± 0.002
Adrenal gland-right (g)	0.039 ± 0.005	0.046 ± 0.006	0.050 ± 0.006	0.045 ± 0.004
Per body weight (%)	0.018 ± 0.002	0.022 ± 0.002	0.022 ± 0.003	0.021 ± 0.001
Uterus (g)	0.62 ± 0.28	0.68 ± 0.24	0.53 ± 0.07	0.84 ± 0.34
Per body weight (%)	0.29 ± 0.14	0.32 ± 0.13	0.24 ± 0.03	0.40 ± 0.16
Ovary-left (g)	0.061 ± 0.010	0.059 ± 0.013	0.065 ± 0.011	0.058 ± 0.006
Per body weight (%)	0.028 ± 0.004	0.028 ± 0.006	0.029 ± 0.002	0.027 ± 0.003
Ovary-right (g)	0.048 ± 0.004	0.062 ± 0.018	0.061 ± 0.013	0.058 ± 0.016
Per body weight (%)	0.022 ± 0.001	0.029 ± 0.005	0.027 ± 0.004	0.028 ± 0.007

Values are presented as means ± S.D.

showed that 28-day repeated oral administration of SUNACTIVE Fe-P80 in rats did not result in any treatment-related adverse effects up to a dose level of 2,000 mg/kg/day, which is considered an acceptable upper limit dose according to ICH guidelines [56]. The results of this study clearly show that a 28-day repeated oral administration of SUNACTIVE Fe-P80, which applies nanotechnology, does not cause any toxic effects in rats.

In conclusion, the study showed that 28-day repeated oral administration of SUNACTIVE Fe-P80 had no significant adverse effects in rats. Under the present experimental conditions, the NOAEL was ≥ 2,000 mg/kg/day in both sexes, and no target organs were identified. Thus, the results obtained from this study suggest that SUNACTIVE Fe-P80 is relatively safe, as no treatment-related adverse effects were observed following a subchronic toxicity experiment. Overall, our results are expected to provide useful information on the general toxic effects of SUNACTIVE Fe-P80 via repeated oral exposure, which can aid in risk assessment.

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Table 9. Histopathological findings of male and female rats in the 28-day repeated oral dose toxicity study of SUNACTIVE Fe-P80

Items	Dose (mg/kg/day)			
	Male		Female	
	0	2,000	0	2,000
No. of animal examined	5	5	5	5
Liver				
Inflammatory cell				
Minimal	1	2	0	0
Focal necrosis				
Minimal	1	0	0	0
Kidney				
Tubular cast				
Minimal	0	0	1	0
Tubular dilation				
Minimal	0	0	0	1
Interstitial inflammatory cell infiltration				
Slight	0	1	0	0
Brain pituitary				
Pituitary, vacuolation				
Minimal	0	1	0	0
Spleen				
EMH				
Minimal	1	1	0	0
Pancreas				
Interstitial inflammatory cell infiltration				
Minimal	0	1	0	0

EMH, extramedullary hematopoiesis.

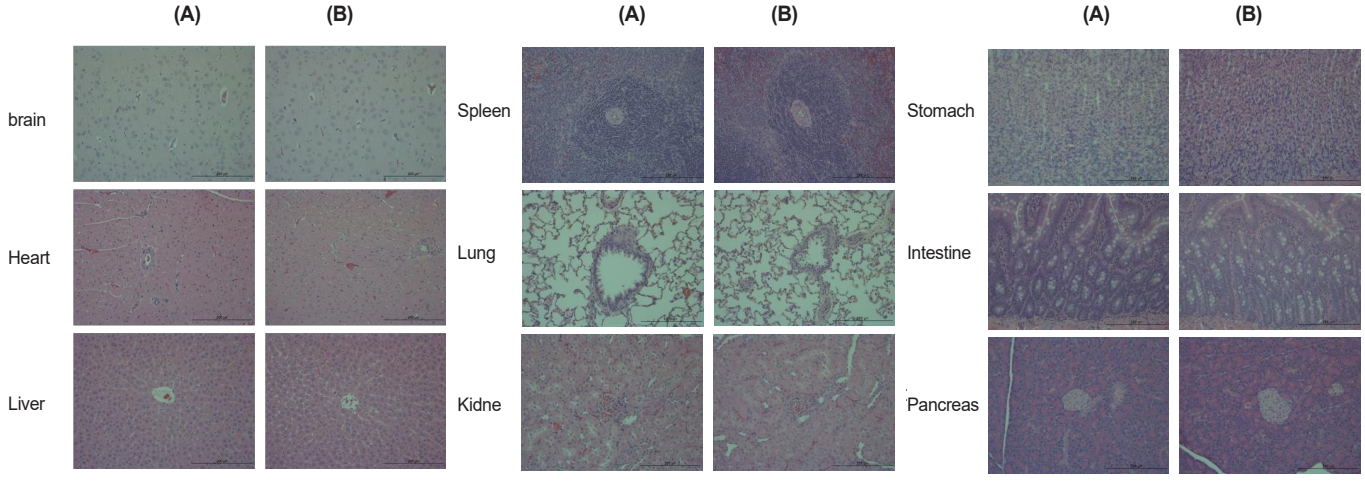

Fig. 1. Histopathological examination of organs from rats after oral SUNACTIVE Fe-P80 treatment for 28 consecutive days. Representative images from male control (A) and high-dose SUNACTIVE Fe-P80 (B) groups. No test substance-induced pathological lesions were noted in the vital organs of either male or female rats (H&E stain, 200 ×).

REFERENCES

1. Crans DC, Woll KA, Prusinskas K, Johnson MD, Norkus E. Metal speciation in health and medicine represented by iron and vanadium. Inorg Chem 2013;52:12262-12275.
2. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. Nat Chem Biol 2014;10:9-17.
3. Sheftel AD, Mason AB, Ponka P. The long history of iron in the universe and in health and disease. Biochim Biophys Acta 2012;1820:161-187.
4. Ross AC, Caballero B, Cousins RJ, Tucker KL, Ziegler TR. Modern nutrition in health and disease. Philadelphia: Lippincott Williams & Wilkins; 2012.
5. Erdman JW Jr, Macdonald IA, Zeisel SH. Present knowledge in nutrition. 10th ed. Hoboken: John Wiley & Sons; 2012.
6. Coates PM, Betz JM, Blackman MR, Cragg GM, Levine M, Moss J, White JD. Encyclopedia of dietary supplements. Boca Raton: CRC Press; 2010.
7. Institute of Medicine (US) Panel on Micronutrients [IOM]. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington: National Academies Press; 2001.
8. World Health Organization [WHO]. Worldwide prevalence of anaemia 1993–2005: WHO global database on anaemia. Geneva: WHO; 2008.
9. Solomons NW. Competitive interaction of iron and zinc in the diet: consequences for human nutrition. J Nutr 1986;116:927-935.
10. Whittaker P. Iron and zinc interactions in humans. Am J Clin Nutr 1998;68:442S-446S.
11. Chang TPY, Rangan C. Iron poisoning: a literature-based review of epidemiology, diagnosis, and management. Pediatr Emerg Care 2011;27:978-985.
12. Manoguerra AS, Erdman AR, Booze LL, Christianson G, Wax PM, Scharman EJ, Woolf AD, Chyka PA, Keyes DC, Olson KR, Martin Caravati E, Troutman WG. Iron ingestion: an evidence-based consensus guideline for out-of-hospital management. Clin Toxicol 2005;43:553-570.
13. Hurrell RF. Fortification: overcoming technical and practical barriers. J Nutr 2002;132:806S-812S.
14. Rossi M, Cubadda F, Dini L, Terranova ML, Aureli F, Sorbo A, Passeri D. Scientific basis of nanotechnology, implications for the food sector and future trends. Trends Food Sci Technol 2014;40:127-148.
15. Ministry of Food and Drug Safety [MFDS]. Good laboratory practice regulation for non-clinical laboratory studies. Notification No. 2017-32. Cheongju: MFDS; 2017.
16. Organisation for Economic Co-operation and Development [OECD]. OECD guidelines for testing of chemicals: repeated dose 28-day oral toxicity study in rodents. Paris: OECD; 2008.
17. National Research Council [NRC]. Guide for the care and use of laboratory animals. Washington: National Academies Press; 2011.
18. Han CT, Kim DY, Nam C, Moon SH, Park SH, Han KG, Lee HY, Bae HM, Park CB, So JH, Kang S, Kang JK. Acute and 13-week subchronic toxicity studies of hot-water extract of

https://doi.org/10.12729/jbtr.2022.23.4.131

Cynanchi wilfordii Radix in Sprague-Dawley rats. Toxicol Res 2020;36:89-98.
19. Lee MJ, Jung HK, Lee KH, Jang JH, Sim MO, Seong TG, Ahn BK, Shon JH, Ham SH, Cho HW, Kim YM, Park SJ, Yoon JY, Ko JW, Kim JC. A 90-day repeated oral dose toxicity study of Alismatis rhizoma aqueous extract in rats. Toxicol Res 2019;35:191-200.
20. Dunnett CW. New tables for multiple comparisons with a control. Biometrics 1964;20:482-491.
21. Kruskal WH, Allen Wallis W. Use of ranks in one-criterion variance analysis. J Am Stat Assoc 1952;47:583-621.
22. Mann HB, Whitney DR. On a test of whether one of two random variables is stochastically larger than the other. Ann Math Stat 1947;18:50-60.
23. Fisher RA. Statistical methods for research workers. In: Kotz S, Johnson NL (eds.). Breakthroughs in statistics. New York: Springer; 1992. p. 66-70.
24. Han ZZ, Xu HD, Kim KH, Ahn TH, Bae JS, Lee JY, Gil KH, Lee JY, Woo SJ, Yoo HJ, Lee HK, Kim KH, Park CK, Zhang HS, Song SW. Reference data of the main physiological parameters in control Sprague-Dawley rats from pre-clinical toxicity studies. Lab Anim Res 2010;26:153-164.
25. Lee JM, Lee MA, Do HN, Song YI, Bae RJN, Lee HY, Park SH, Kang JS, Kang JK. Historical control data from 13-week repeated toxicity studies in Crj:CD (SD) rats. Lab Anim Res 2012;28:115-121.
26. Greaves P. Histopathology of preclinical toxicity studies: interpretation and relevance in drug safety evaluation. 4th ed. Cambridge: Academic Press; 2011.
27. Suttie AW, Leininger JR, Bradley AE. Boorman's pathology of the rat: reference and atlas. Amsterdam: Elsevier; 2017.
28. Wallig MA, Bolon B, Haschek WM, Rousseaux CG. Fundamentals of toxicologic pathology. 3rd ed. Amsterdam: Academic Press; 2017.
29. Elder A, Lynch I, Grieger K, Chan-Remillard S, Gatti A, Gnewuch H, Kenawy E, Korenstein R, Kuhlbusch T, Linker F, MatiasS, Monteiro-Riviere N, Pinto VRS, Rudnitsky R, Savolainen K, Shvedova A. Human health risks of engineered nanomaterials. In: Linkov I, Steevens J (eds.). Nanomaterials: risks and benefits. Dordrecht: Springer; 2009. p. 3-29.
30. Savolainen K, Alenius H, Norppa H, Pylkkänen L, Tuomi T, Kasper G. Risk assessment of engineered nanomaterials and nanotechnologies: a review. Toxicology 2010;269:92-104.
31. Barnes CA, Elsaesser A, Arkusz J, Smok A, Palus J, Leśniak A, Salvati A, Hanrahan JP, de Jong WH, Dziubałtowska E, Stępnik M, Rydzyński K, McKerr G, Lynch I, Dawson KA, Vyvyan Howard C. Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity. Nano Lett 2008;8:3069-3074.
32. Kisin ER, Murray AR, Keane MJ, Shi XC, Schwegler-Berry D, Gorelik O, Arepalli S, Castranova V, Wallace WE, Kagan VE, Shvedova AA. Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells. J Toxicol Environ Health A 2007;70:2071-2079.
33. Heng BC, Zhao X, Tan EC, Khamis N, Assodani A, Xiong S, Ruedl C, Ng KW, Loo JSC. Evaluation of the cytotoxic and inflammatory potential of differentially shaped zinc oxide nanoparticles. Arch Toxicol 2011;85:1517-1528.

34. Jones CF, Grainger DW. *In vitro* assessments of nanomaterial toxicity. Adv Drug Deliv Rev 2009;61:438-456.
35. Karlsson HL, Gustafsson J, Cronholm P, Möller L. Size-dependent toxicity of metal oxide particles: a comparison between nano- and micrometer size. Toxicol Lett 2009;188:112-118.
36. Khan MI, Mohammad A, Patil G, Naqvi SAH, Chauhan LKS, Ahmad I. Induction of ROS, mitochondrial damage and autophagy in lung epithelial cancer cells by iron oxide nanoparticles. Biomaterials 2012;33:1477-1488.
37. Xie G, Sun J, Zhong G, Shi L, Zhang D. Biodistribution and toxicity of intravenously administered silica nanoparticles in mice. Arch Toxicol 2010;84:183-190.
38. Botelho MC, Costa C, Silva S, Costa S, Dhawan A, Oliveira PA, Teixeira JP. Effects of titanium dioxide nanoparticles in human gastric epithelial cells *in vitro*. Biomed Pharmacother 2014;68:59-64.
39. Sanders K, Degn LL, Mundy WR, Zucker RM, Dreher K, Zhao B, Roberts JE, Boyes WK. *In vitro* phototoxicity and hazard identification of nano-scale titanium dioxide. Toxicol Appl Pharmacol 2012;258:226-236.
40. Valdiglesias V, Costa C, Sharma V, Kiliç G, Pásaro E, Teixeira JP, Dhawan A, Laffon B. Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. Food Chem Toxicol 2013;57:352-361.
41. Chen J, Patil S, Seal S, McGinnis JF. Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. Nat Nanotechnol 2006;1:142-150.
42. Dumortier H, Lacotte S, Pastorin G, Marega R, Wu W, Bonifazi D, Briand JP, Prato M, Muller S, Bianco A. Functionalized carbon nanotubes are non-cytotoxic and preserve the functionality of primary immune cells. Nano Lett 2006;6:1522-1528.
43. Gupta AK, Gupta M. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. Biomaterials 2005;26:3995-4021.
44. Wilhelm C, Billotey C, Roger J, Pons JN, Bacri JC, Gazeau F. Intracellular uptake of anionic superparamagnetic nanoparticles as a function of their surface coating. Biomaterials 2003;24:1001-1011.
45. Lockman PR, Koziara JM, Mumper RJ, Allen DD. Nanoparticle surface charges alter blood–brain barrier integrity and permeability. J Drug Target 2004;12:635-641.
46. Lemarchand C, Gref R, Lesieur S, Hommel H, Vacher B, Besheer A, Maeder K, Couvreur P. Physico-chemical characterization of polysaccharide-coated nanoparticles. J Control Release 2005;108:97-111.
47. Schrand AM, Braydich-Stolle LK, Schlager JJ, Dai L, Hussain SM. Can silver nanoparticles be useful as potential biological labels? Nanotechnology 2008;19:235104.
48. Goodman CM, McCusker CD, Yilmaz T, Rotello VM. Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. Bioconjug Chem 2004;15:897-900.
49. Wagner AJ, Bleckmann CA, Murdock RC, Schrand AM, Schlager JJ, Hussain SM. Cellular interaction of different forms of aluminum nanoparticles in rat alveolar macrophages. J Phys Chem B 2007;111:7353-7359.
50. Bianco A, Kostarelos K, Partidos CD, Prato M. Biomedical applications of functionalised car-

 https://doi.org/10.12729/jbtr.2022.23.4.131

bon nanotubes. Chem Commun 2005:571-577.

51. Chen C, Xing G, Wang J, Zhao Y, Li B, Tang J, Jia G, Wang T, Sun J, Xing L, Yuan H, Gao Y, Meng H, Chen Z, Zhao F, Chai Z, Fang X. Multihydroxylated $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles: antineoplastic activity of high efficiency and low toxicity. Nano Lett 2005;5:2050-2057.
52. Pantarotto D, Briand JP, Prato M, Bianco A. Translocation of bioactive peptides across cell membranes by carbon nanotubes. Chem Commun 2004;1:16-17.
53. Pantarotto D, Partidos CD, Graff R, Hoebeke J, Briand JP, Prato M, Bianco A. Synthesis, structural characterization, and immunological properties of carbon nanotubes functionalized with peptides. J Am Chem Soc 2003;125:6160-6164.
54. Perez JM, Asati A, Nath S, Kaittanis C. Synthesis of biocompatible dextran-coated nanoceria with pH-dependent antioxidant properties. Small 2008;4:552-556.
55. Schrand AM, Huang H, Carlson C, Schlager JJ, Ōsawa E, Hussain SM, Dai L. Are diamond nanoparticles cytotoxic? J Phys Chem B 2007;111:2-7.
56. European Medicines Agency [EMA], Committee for Proprietary Medicinal Products [CPMP], International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [ICH]. ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. Amsterdam: European Medicines Agency; 2009.