**In vitro** genotoxicity assessment and 28-day repeated dose oral toxicity study of steady-calcium formula in rats

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**A B S T R A C T**

Steady-calcium formula (SCF), a functional food mixture with potential of joint care, contains five major ingredients. However, the uncertain cross-reactivity among these included ingredients cannot be excluded. Hence, it is important to ensure the safety of this mixture. In this study, the safety of SCF was evaluated through in vitro genotoxicity assessment and 28-day oral toxicity study in rats. The bacterial reverse mutation test and mammalian chromosome aberration test displayed that SCF did not induce mutagenicity and clastogenicity. The 28-day repeated dose assessment of SCF in rats revealed no mortality and adverse effects in clinical signs, body weight, urinalysis, hematology, organ weight, and histopathology at all treated groups. Although some significant changes were observed in food intake and parameters of serum biochemistry at the highest dose in males, they were not dose-related and considered to be within normal range. These findings indicate that SCF does not possess genotoxic potential and no obvious evidence of subacute toxicity. These results demonstrate for the first time that the genotoxicity and subacute toxicity for SCF are negative under our experimental conditions and the no observed adverse effect level (NOAEL) of SCF may be defined as at least 5470 mg/kg/day.

1. Introduction

Functional foods are known to possess a significant potential on health beyond normal nutrition. Steady-calcium formula (SCF) is a type of functional food in Taiwan containing hydrolyzed chicken sternal cartilage extract, calcium citrate, mangosteen extract, turmeric (*Curcuma longa*) extract and soybean extract. Several studies have demonstrated that collagen hydrolysates can increase biosynthesis of type II collagen, which is the major type in cartilage and can effectively prevent bone deterioration in a tail-suspension simulated microgravity rat model [1–4]. A randomized, double-blind study has shown that collagen hydrolysates administration leads to reduction in joint pain [5]. A clinical trial has also observed a significant reduction in knee osteoarthritic pain with collagen hydrolysates intake, which contributes to maintain the function of cartilage [6]. Daily supplementation with calcium citrate helped fracture healing in a femur defect rabbit model [7]. Combination with calcium and vitamin D has been found to reduce bone loss in human 65 years of age or older [8]. Moreover, combined collagen hydrolysates and calcium citrate has been found to reduce bone loss in ovariectomized rats [9] and improved the bone deterioration in a tail-suspension simulated microgravity rat model [10]. Mangosteen is a traditional medicine, which contains α-mangostin and possesses multiple biological activities, such as antioxidant, anti-cancer, anti-apoptosis, anti-inflammation, antibacterial, anti-obesity, and nephro-protective effects [11–14]. α-Mangostin suppressed the activity of key enzymes involved in cholesterol and triglyceride metabolism, such as HMG-CoA reductase and lipoprotein lipase.

**Abbreviations:** ALK. Phos., alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CHO, Chinese hamster ovary; CPK, creatine phosphate kinase; γ-GT, gamma glutamyl transpeptidase; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MOS, margin of safety; MPV, mean platelet volume; NOAEL, no observed adverse effect level; OECD, organization for economic cooperation and development; PT, prothrombin time; RBC, red blood cell; RDW, red blood cell volume distribution width; SCF, steady-calcium formula; TFDA, Taiwan food and drug administration; WBC, white blood cell.

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https://doi.org/10.1016/j.toxrep.2022.04.008
Received 13 December 2021; Received in revised form 19 March 2022; Accepted 12 April 2022
Available online 16 April 2022
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Toxicology Reports 9 (2022) 834–841
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degeneration of cartilage through inhibition of interleukin-1β signaling in osteoarthritis rat model [15]. Turmeric extract contains a potent antioxidant curcumin, which possesses numerous pharmacological effects to be used as a preventive and therapeutic agent in multi-diseases including osteoporosis. It has been shown that turmeric extract prevents osteoclasts differentiation and loss of bone density [16,17]. Soy isoflavones from soybeans are well known polyphenol compounds with potent estrogenic, anti-inflammatory, and anti-oxidative activities. Soy isoflavones have a potential to arrest bone loss in ovariectomized rats [18] and elevated bone turnover, bone mineral density and bone mechanical strength in postmenopausal women [19,20]. In addition, combination of soy isoflavones and calcium possesses a potential on retarding bone mineral density loss in perimenopausal women [21]. It is majorly concerning that the ingredients in mixtures of herbal medicinal drugs or functional foods can induce the cocktail effects and synergistic interactions, particularly in relation to toxic responses [22]. Despite the efficacy, pharmacology, and safety of each ingredient of SCF has been verified, the interaction and synergistically toxicological effects are needed to be concerned. Therefore, this study aimed to evaluate the safety profile of SCF using in vitro genotoxicity assays of bacteria and mammalian cells and 28-day repeated oral dose toxicity test in rats.

2. Materials and methods

2.1. Test substances

The test substance SCF is a yellow powder. Its package as a dietary supplement is 30 tablets per pack and 4 packs per box. A human recommended daily intake for SCF is 4 tablets/day (820 mg/tablet). It contains hydrolyzed chicken sternal cartilage extract (30.5%), calcium citrate (24.4%), mangosteen (Garcinia mangostana) extract (12.2%), turmeric (Curcuma longa) extract (5.5%) and soybean extract (3%). It was provided by HealthTake Corporation (Taichung, Taiwan). The

Table 1
Bacterial reverse mutation test of SCF.

| Treatment (Without S9 mix) | Mean revertant colonies per plate |
|----------------------------|----------------------------------|
| TA98 | TA100 | TA1535 | TA1537 | WP2 uvrA/pKM101 |
| Negative Control | 20 ± 4 * | 79 ± 4 | 10 ± 3 | 4 ± 2 | 104 ± 6 |
| Positive Control | 76 ± 1 * | 452 ± 38 * | 1600 ± 413 * | 32 ± 7 * | 598 ± 28 * |
| 5 mg | 24 ± 6 | 82 ± 13 | 11 ± 7 | 5 ± 2 | 123 ± 23 |
| 2.5 mg | 22 ± 1 | 88 ± 6 | 9 ± 4 | 4 ± 1 | 126 ± 28 |
| 1.25 mg | 20 ± 4 | 83 ± 10 | 16 ± 5 | 4 ± 2 | 102 ± 5 |
| 0.6 mg | 25 ± 3 | 74 ± 13 | 11 ± 3 | 6 ± 3 | 96 ± 17 |
| 0.3 mg | 23 ± 5 | 77 ± 10 | 9 ± 4 | 5 ± 1 | 100 ± 11 |

Data are presented as mean ± S.D. of three independent experiments done in triplicate. * P < 0.05 vs negative control.

Table 2
Chromosome aberration test with SCF in CHO-K1 cells.

| Treatment (± S9 mix, 3 h) | Aberrant cells (%) | No. of chromosome aberrations for total 300 cells |
|---------------------------|--------------------|--------------------------------------------------|
| TA98 | TA100 | TA1535 | TA1537 | WP2 uvrA/pKM101 |
| Negative control | 0.67 | 1 0 1 1 0 0 0 0 | | | |
| Positive control | | 4 | 11 2 3 2 3 4 0 | | |
| 0.0375 (mg/mL) | 1.33 | 0 1 0 0 0 2 1 0 | | |
| 0.075 (mg/mL) | 0 | 0 0 0 0 0 0 0 0 | | |
| 0.15 (mg/mL) | 0.33 | 1 0 1 1 0 1 0 0 | | |
| 0.3 (mg/mL) | 1.67 | 0 1 1 1 1 2 0 0 | | |
| 0.6 (mg/mL) | 1 | 0 0 1 0 3 2 0 0 | | |
| Negative control | 0 | 1 0 0 0 0 0 0 0 | | |
| Positive control | 2 | 12 0 1 1 5 3 1 0 | | |
| 0.0375 (mg/mL) | 0.33 | 1 1 0 0 3 0 0 0 | | |
| 0.075 (mg/mL) | 0.33 | 2 0 0 0 0 0 1 0 | | |
| 0.15 (mg/mL) | 0.67 | 0 0 0 0 2 2 0 0 | | |
| 0.3 (mg/mL) | 0.33 | 2 0 0 0 2 1 0 0 | | |
| 0.6 (mg/mL) | 0.67 | 1 0 0 0 3 2 0 0 | | |
| Negative control | 1.33 | 1 0 2 0 0 1 1 0 | | |
| Positive control | 5.67 | 4 4 5 4 2 7 1 0 | | |
| 0.0375 (mg/mL) | 0.33 | 1 0 0 0 2 0 1 0 | | |
| 0.075 (mg/mL) | 0.33 | 0 0 1 0 1 0 0 0 | | |
| 0.15 (mg/mL) | 1 | 1 0 1 0 0 2 0 0 | | |
| 0.3 (mg/mL) | 0.33 | 1 0 0 1 0 0 0 0 | | |
| 0.6 (mg/mL) | 1.67 | 2 0 1 0 0 4 0 0 | | |

a Aberrant cells were calculated excluding cells with gaps.

b Positive control was 0.07 μg/mL mitomycin C. Data was recorded independently by two groups of observers.

c Chromosome aberration test with SCF in CHO-K1 cells.

2. Materials and methods

2.1. Test substances

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Table 3
Effects of SCF on body weights in a 28-day oral toxicity test in rats.

| Male Observation time | SCF (mg/kg/day) |
|-----------------------|----------------|
| Vehicle*             | 1640 | 3280 | 5470 |
| Week 0 | 250.7 ± 15.7 | 254.1 ± 9.6 | 254.8 ± 8.5 | 259.9 ± 11.6 |
| Week 1 | 314.1 ± 16.9 | 309.4 ± 13.4 | 309.0 ± 9.9 | 310.8 ± 15.3 |
| Week 2 | 359.2 ± 18.9 | 346.3 ± 15.9 | 352.1 ± 13.4 | 350.8 ± 21.1 |
| Week 3 | 494.4 ± 23.4 | 385.4 ± 19.0 | 390.3 ± 16.3 | 387.9 ± 25.3 |
| Week 4 | 432.3 ± 26.7 | 413.1 ± 25.7 | 419.8 ± 24.0 | 413.6 ± 22.9 |

| Female Observation time | SCF (mg/kg/day) |
|-------------------------|----------------|
| Vehicle*                | 1640 | 3280 | 5470 |
| Week 0 | 171.6 ± 6.7 | 170.6 ± 6.5 | 173.2 ± 8.6 | 172.7 ± 5.5 |
| Week 1 | 190.5 ± 8.6 | 185.9 ± 11.2 | 185.7 ± 8.7 | 188.9 ± 8.8 |
| Week 2 | 208.2 ± 4.3 | 197.8 ± 11.0 | 207.9 ± 13.4 | 202.5 ± 6.8 |
| Week 3 | 222.3 ± 6.6 | 209.8 ± 12.3 | 220.2 ± 17.0 | 216.7 ± 7.6 |
| Week 4 | 236.7 ± 9.1 | 224.4 ± 11.1 | 231.8 ± 16.2 | 228.4 ± 9.6 |

Data are presented as mean±S.D. (n = 10). * Distilled water.
Table 4

| Effects of SCF on food intake in a 28-day oral toxicity test in rats. |
|-------------------|---|---|---|
|                  | Male (g) SCF (mg/kg/day) |       |     |
|                   | Vehicle | 1640 | 3280 | 5470 |
| Week 1            | 27.6 ± 1.0 | 26.4 ± 1.2 | 26.6 ± 0.8 | 25.1 ± 1.7 |
| Week 2            | 31.2 ± 1.1 | 29.8 ± 2.0 | 29.3 ± 0.8 | 26.7 ± 2.1 *
| Week 3            | 30.2 ± 2.9 | 30.4 ± 2.0 | 30.1 ± 1.9 | 28.6 ± 1.7 |
| Week 4            | 27.3 ± 0.9 | 25.9 ± 2.2 | 26.3 ± 2.4 | 24.3 ± 2.3 |
| Female (g)        | SCF (mg/kg/day) |       |     |
|                   | Vehicle | 1640 | 3280 | 5470 |
| Week 1            | 17.7 ± 1.3 | 16.6 ± 0.2 | 16.0 ± 1.1 | 14.4 ± 1.7 |
| Week 2            | 18.9 ± 0.9 | 17.9 ± 0.8 | 18.8 ± 1.3 | 17.4 ± 0.5 |
| Week 3            | 18.6 ± 0.9 | 17.1 ± 0.2 | 17.9 ± 1.1 | 18.0 ± 0.7 |
| Week 4            | 16.4 ± 0.8 | 15.3 ± 0.3 | 15.3 ± 1.2 | 15.1 ± 0.4 |

Data are presented as mean±S.D. (n = 10). * P < 0.05 vs vehicle.

Various extracts in SCF were prepared by HealthTake Corporation. The methods for preparation of these extracts were described briefly as follows. For preparation of hydrolyzed chicken stern al cartilage extract, the pepsin-assisted extraction method was used. The grinding chicken stern al cartilage was dissolved in water, and then added pepsin for digesting the insoluble matter. Samples were centrifuged and then the supernatants were collected. Added 1 M Tris-base to salt out the supernatants, and then centrifuged to obtain the precipitates. The precipitates were dissolved in 0.1 M acetic acid, dialyzed against distilled water, and then lyophilized. For preparation of mangosteen extract, the fruits and leaf of mangosteen were dried and ground to powder, then it was mixed and percolated with 85% ethanol. The extraction was evaporated by a rotation evaporator until achieving the extract. Preparation of turmeric extract, the dried turmeric samples were extracted with absolute ethanol (99.9%), and then lyophilized. For preparation of soybean extract, the soy flour was extracted with 80% ethanol at room temperature, and then lyophilized.

During experiments, SCF was dissolved in distilled water freshly before use. The same batch (batch number: HT-HFT200605) of SCF was used to perform the genotoxicity assay and 28-day oral toxicity test.

2.2. In vitro bacterial reverse mutation test (Ames test)

The *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and a tryptophan-requiring *E. coli* mutant WP2 uvrA/pKM101 (Excel Biomedical INC., Taipei, Taiwan) were used as previously described by Maron and Ames [23] and the guidelines of Organization for Economic Cooperation and Development (OECD; TG-471) [24] and Taiwan Food and Drug Administration (TFDA; 2020) [25].

The rat liver S9 fraction for metabolic activation was prepared from rats induced by 3-methylcholanthrene as previously described [26]. Rat liver was washed, homogenized, and then centrifuged for 10 min at 9000 × g. Collected the supernatant (S9 fraction) and then stored at –80 °C. The 0.5 mL S9 mix (0.05 mL S9 fraction with 0.45 mL cofactors: MgCl2 (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM), NADPH (4 mM), NADH (4 mM), and sodium phosphate (100 mM; pH7.4)) was prepared freshly before performing the tests.

The plate incorporation and preincubation modification procedures using bacterial tester strains with and without S9 metabolic activation were performed. The tested concentrations were set at 5.0, 2.5, 1.25, 0.6, and 0.3 mg/plate, which were recommended for soluble non-cytotoxic substances by the guidelines of the OECD and TFDA. Briefly, added 0.2 mL of 0.5 M histidine/biotin (for TA98, TA100, TA1535, and TA1537) or 0.37 M tryptophan (for *E. coli* WP2 uvrA/pKM101) to the top agar and distributed 2 mL of top agar into tubes and incubated under 45 °C. And then added 0.1 mL of test substance and 0.1 mL of overnight incubated bacterial strain into the top agar, mixed, and poured onto a glucose minimal agar plate. After incubation at 37 °C for 48 h, the numbers of revertant colonies were counted. In the S9-treated groups, the positive controls were used: 5 μg/plate of benzo[a]pyrene for TA98; 2-aminoanthracene for TA100, TA1535, TA1537 strains (8 μg/plate) and WP2 uvrA/pKM101 strain (20 μg/plate). Under conditions without S9 treatment, the positive controls were used: 0.5 μg/plate of 4-nitroquinoline-N-oxide for TA98 strain; Sodium azide (4 μg/plate) for TA100 and TA1535 strains; 9-aminoacridine (5 μg/plate) for TA1537; 4-nitroquinoline-1-oxide (0.2 μg/plate) for WP2 uvrA/pKM101. Data from three independent experiments, each performed in triplicate, are presented. Results were represented as number of revertant colonies per plate and concentration and by the mutation rate. The positive response was determined that the numbers of revertants observed in the test substance treatment group showed more than two-fold compared to the negative control group for TA98, TA100, and WP2 uvrA/pKM101 strains, and more than three-fold compared to the negative control group for TA1535 and TA1537 strains.

2.3. Mammalian chromosomal aberration in vitro test

According to the guidelines from OECD (TG-473) [27] and TFDA (2020) [25], the *in vitro* mammalian chromosomal aberrations test was used to assess whether SCF caused structural chromosomal aberrations in cultured mammalian cells with and without S9 metabolic activation. Chinese hamster ovary (CHO-K1) cells were purchased from the Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). CHO-K1 cells (4 × 10⁵) were seeded in 60 mm dishes for 24 h and treated with different doses of test substances. The highest dose was determined at the level that would inhibit up 50% cell growth compared to control by cytoxicity assay using a Cell Counting Kit-8 (MilliporeSigma, St. Louis, MO, USA). The final concentrations of SCF used in this assay were 0.6, 0.3, 0.15, 0.075, and 0.0375 mg/mL. Tests were divided into three groups: (1) test substance without S9 mix for 3 h, (2) test substance with S9 mix for 3 h, and (3) test substance without S9 mix for 20 h. In the groups of (1) and (2), the media were changed after 3 h, and then cells were cultured for 17 h (total 20 h). Mitomycin C (MilliporeSigma; 0.07 μg/mL) was used as a positive control without S9 mix for both 3 and 20 h. Cyclophosphamide monohydrate (MilliporeSigma; 10 μg/mL) was used as a positive control with S9 mix for 3 h. After 20 h of treatment, cells were treated with Colcemid (MilliporeSigma; 0.1 μg/mL) for 2 h to block mitosis at metaphase, and then Giemsa solution (5%) was used for staining. Chromosome aberrations were observed under 1000 × magnification in 100 well-spread metaphases per concentration of test substance performed in triplicate. The percentage of structural chromosomal abnormalities, including acentric fragment, chromatid gap, chromatid break, chromosome gap, chromosome break, dicentric, multiple aberrations, and ring were determined. The gaps were reported separately but not included in the total aberration frequency. When the chromosome...
aberrant cells (%) in SCF-treated group showed statistically significant difference (P < 0.05) compared to the negative control, the result of test substance was considered to be positive.

2.4. Animals

Wistar rats (5–6-week-old, the average body weight was 255 g in male rats and 172 g in female rats) of each sex (10 rats/group/sex) were provided by BioLasco Taiwan Co. Ltd., Taipei, Taiwan. The protocol for animal study was approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Centre at National Taiwan University College of Medicine, Taipei, Taiwan. Rats were maintained in accordance with the Guide of Association for Assessment and Accreditation of Laboratory Animal Care International. Rats were fed with standard diets and water ad libitum, acclimatized for one week, and housed in cages under controlled temperature (22 ± 3°C) and relative humidity of 55 ± 15% with 12 h/12 h light/dark cycle.

2.5. Repeated dose 28-day oral toxicity study

The 28-day oral toxicity study in rats was performed according to the guidelines of OECD (TG-407) [28] and TFDA (2020) [25]. Rats were divided into four groups, including control, 1640 mg/kg (low-dose group), 3280 mg/kg (middle-dose group), and 5470 mg/kg (high-dose group) via oral gavage once daily for 28 consecutive days. Distilled water was used as a vehicle control. The dose volume for each group was 10 mL/kg. General behavior, clinical symptoms, and toxic signs were observed daily during the experimental period. Body weight and food consumption were monitored and recorded twice a week. At the end of study, all animals were housed in metabolic cages and fasted overnight for collection of urine samples. Subsequently, the rats were anesthetized with inhalation of isoflurane (3%) and blood samples were collected from abdominal aorta for hematology and serum biochemical analysis. The organs/tissues of adrenal glands, brain, epididymis, heart, kidney, liver, lung, ovary, spleen, thymus, testis, and uterus were immediately weighted, and the relative weights of organs were normalized with terminal body weights.

The dosage of SCF was determined following the regulation for health food required by TFDA based on the concept of margin of safety (MOS) [25,29]. A human recommended daily intake for SCF recognized as a functional food has been suggested to be 54.7 mg/kg/day for a 60 kg person by supplier. To determine the no observed adverse effect level (NOAEL) for SCF, three doses of 1640, 3280, and 5470 mg/kg were tested, corresponding to the 30-, 60- and 100-fold exposure of the human recommended daily intake, respectively.

2.6. Urinalysis, hematology, and serum biochemical analysis

Urine was collected overnight under fasting condition and analyzed the following parameters using the CLINITEK Status+ analyzer (Siemens Medical Solutions Diagnostics Inc., Los Angeles, USA) or directly visual measurement, including color, glucose, bilirubin, ketone, specific gravity, protein, pH, uricinogen, nitrite, leukocyte, and occult blood.
Data are presented as mean ± S.D. (n = 10). * P < 0.05 vs vehicle.

3. Results and discussion

The test substance SCF contained five major ingredients possessed the potential with joint care. Despite the safety assessment of hydrolyzed chicken sternal cartilage extract, calcium citrate, mangosteen extract, turmeric extract and soybean extract by themselves have been investigated [30–33], the genotoxicity and 28-day oral toxicity of SCF containing them have not been verified. However, the uncertain cross-reactivity among these included ingredients cannot be excluded. It is important to ensure the safety of SCF. Therefore, the safety of SCF was evaluated through in vitro genotoxicity assessment and 28-day oral toxicity study in rats.

3.1. Genotoxicity

As shown in Table 1, SCF (0.3, 0.6, 1.25, 2.5, 5.0 mg/plate) did not significantly enhance the revertant mutation colonies for any strains either in the presence or absence of S9 mixtures. The average number of revertant colonies was within the normal range in the negative control either in the presence or absence of S9 mixtures. The average number of revertant colonies was within the normal range in the negative control.
contrast, the positive control groups markedly showed abnormal structural aberrations, including gaps, breaks, dicentrics, rings, and multiple aberrations (Table 2). Thus, the result of the mammalian chromosomal aberrations test with SCF was negative under the experimental conditions of this study.

Numerous studies have evaluated the toxicity among these included ingredients. For instance, soy isoflavones by themselves had no effects on the induction of bone marrow chromosomal aberration and micronuclei in vivo, but could antagonize the benzo[a]pyrene-induced genotoxicity [31]. The leaf ethanol extracts of mangosteen have been found to induce genotoxicity and apoptosis in B16-F10 melanoma cells [30]. In contrast, our genotoxicity results suggest that the mixture of these ingredients, including the leaf ethanoic extracts of mangosteen in SCF does not induce genotoxicity. A previous study for in vitro genotoxicity tests has shown that turmeric (Curcuma longa) polysaccharide extract is genotoxically safe [33]. UC-II, an undenatured native chicken

Table 7
Effects of SCF on relative organ/tissue weights in a 28-day oral toxicity test in rats.

|            | SCF (mg/kg/day) | Vehiclea | 1640 | 3280 | 5470 |
|------------|-----------------|----------|------|------|------|
| Heart      | 2877.7 ± 243.4  | 2894.7 ± 147.5 | 2802.4 ± 311.6 | 2917.0 ± 213.9 |
| Liver      | 29398.2 ± 2990.3| 29007.8 ± 1408.2 | 30128.5 ± 2489.9 | 30250.5 ± 1964.7 |
| Spleen     | 2507.5 ± 347.0  | 2273.4 ± 204.0  | 2290.6 ± 259.2  | 2272.2 ± 202.7  |
| Adrenal Gland| 164.7 ± 34.3    | 177.4 ± 25.9    | 175.9 ± 27.6    | 181.3 ± 18.8    |
| Kidney     | 6848.4 ± 446.1  | 7131.2 ± 488.1  | 7231.0 ± 519.7  | 7393.5 ± 564.9  |
| Epididymis | 2175.5 ± 354.0  | 2209.2 ± 213.8  | 2177.3 ± 186.9  | 2408.3 ± 274.0  |
| Testis     | 8276.8 ± 848.8  | 8566.6 ± 806.7  | 8287.0 ± 790.4  | 9056.0 ± 910.2  |
| Lung       | 3459.3 ± 244.1  | 3489.6 ± 206.6  | 3404.1 ± 332.3  | 3645.2 ± 261.6  |
| Thymus     | 1460.3 ± 256.6  | 1450.1 ± 182.4  | 1228.8 ± 193.9  | 1331.0 ± 244.6  |
| Brain      | 5050.9 ± 368.6  | 5100.0 ± 485.0  | 5070.7 ± 450.2  | 5384.5 ± 562.3  |

|            | SCF (mg/kg/day) | Vehiclea | 1640 | 3280 | 5470 |
|------------|-----------------|----------|------|------|------|
| Heart      | 3212.4 ± 144.5  | 3126.3 ± 171.7 | 3179.3 ± 216.0 | 3100.3 ± 200.0 |
| Liver      | 30767.2 ± 2990.1| 31310.4 ± 2801.1| 31512.0 ± 2009.7| 32137.6 ± 1533.1|
| Spleen     | 2367.6 ± 310.0  | 2514.7 ± 263.8 | 2507.6 ± 289.3 | 2568.6 ± 171.5 |
| Adrenal Gland| 335.2 ± 33.2    | 328.7 ± 57.9   | 359.9 ± 34.6   | 378.2 ± 49.4   |
| Kidney     | 6932.7 ± 560.2  | 7025.7 ± 312.5 | 7185.5 ± 343.0 | 7074.6 ± 295.2 |
| Ovary      | 594.8 ± 81.3    | 576.1 ± 61.7   | 586.5 ± 57.9   | 628.7 ± 98.8   |
| Uterus     | 2083.4 ± 437.7  | 2620.3 ± 909.4 | 2462.2 ± 617.5 | 2256.2 ± 598.7 |
| Lung       | 4488.7 ± 247.8  | 4588.8 ± 230.6 | 4485.2 ± 116.8 | 4425.8 ± 216.8 |
| Thymus     | 1688.3 ± 279.4  | 1544.2 ± 403.4 | 1733.3 ± 305.1 | 1725.0 ± 850.8 |
| Brain      | 8218.0 ± 264.8  | 8605.5 ± 495.5 | 8504.9 ± 545.4 | 8654.4 ± 421.2 |

Data are presented as mean ± S.D. (n = 10 per group).

Fig. 1. Histopathological examinations in male and female rats treated with SCF. No significantly histopathological changes were found in the adrenal gland (a, a’), epididymis (b, b’), heart (c, c’), kidney (d, d’), liver (e, e’), spleen (f, f’), testis (g, g’), ovary (h, h’), oviduct (i, i’), uterus (j, j’), and cervix (k, k’) in female rats by H&E staining. The vehicle control group was shown in a–k. The high-dose group was shown in a’–k’. Scale bar = 100 µm.
type II collagen produced from chicken sternum cartilage, has been shown that it did not induce the mutagenic effects in the bacterial reverse mutation test and in the mouse lymphoma cell gene mutation test [34]. In the present study, the results of in vitro genotoxicity tests suggest that the mixture of these ingredients in SCF does not induce mutagenicity and clastogenicity.

3.2. Repeated dose 28-day oral toxicity

No obvious clinical abnormalities or deaths were observed in any groups during the 28-day oral toxicity study. Mean body weights were not significantly different among SCF-treated and control groups (Table 3), although the food intake in the high-dose (5470 mg/kg) group was lower than control group in males at the second week (Table 4). Since this change was occasional and did not show dose-dependent manner, it was not considered to be test substace-related.

The results of urinary parameters showed no significantly different among SCF-treated and control groups in either male or female rats (Table 5). The data for hematological parameters of the SCF-treated and control groups were shown in Table 6. No significant differences in the hematological parameters were observed for the SCF-treated groups compared to the control group in either male or female rats. Moreover, the results of serum biochemistry showed that there were several parameters significantly decreased in high dose SCF-treated male rats, including SGOT/AST (P = 0.0027), total protein (P < 0.0001), albumin (P < 0.0001), globulin (P = 0.0013), creatinine (P = 0.006), cholesterol (P = 0.0004), Ca^{2+} (P < 0.0001), P (P = 0.0029), and CPK (P = 0.049) compared to the control male rats (Table 6). Nevertheless, these significant changes of serum biochemistry in high-dose SCF-treated male rats are considered incidental. The dose-dependent manner and adverse clinical abnormalities are not observed in high-dose SCF-treated male rats during the study. Since these changes in high-dose SCF-treated male rats were within the normal range from our Lab (SGOT/AST: 72.5–304 U/L; total protein: 5–7.1 g/dl; albumin: 3.35–5.2 g/dl; globulin: 1.1–2.5 g/dl; creatinine: 0.22–0.72 mg/dl; cholesterol: 34.1–108.1 mg/dl; Ca^{2+}: 2.37–14.63 mg/dl; P: 5.6–17.59 mg/dl; CPK: 502–1526) and Charles River Laboratories [35] (SGOT/AST: 74–143 U/L; total protein: 5.2–7.1 g/dl; albumin: 3.4–4.8 g/dl; globulin: 1.5–2.5 g/dl; creatinine: 0.2–0.5 mg/dl; cholesterol: 37–85 mg/dl; Ca^{2+}: 9.5–11.5 mg/dl; P: 5.58–10.41 mg/dl; CPK: 162–1184), and did not show dose-dependent manner, these were not SCF-related alterations. No significant differences in the parameters of serum biochemistry were observed in the SCF-treated female rats compared to the control female rats (Table 6).

The organ weight data displayed that there were no significant changes in relative organ weights (absolute organ weight normalized with body weight) for adrenal glands, brain, epididymis, heart, kidney, liver, lung, ovary, spleen, thymus, testis, and uterus among SCF-treated and control groups in both sex (Table 7). In addition, the histological examination with heart, liver, spleen, kidney, adrenals gland, testis, epididymis, ovary and uterus in high-dose group was not observed any obvious lesions and serious aberrations compared to the control groups in both sex (Fig. 1).

Several studies have assessed the toxicity of the ingredients of SCF. The NOAEL for a hydrolyzed chicken sternal cartilage preparation has been estimated to be 30 mg/kg in a 90-day repeated dose subchronic toxicity test [32]. A 4-year randomized clinical trial has shown that calcium citrate supplementation at the dose of 670 mg/day significantly increases the bone accretion in young women [36]. The lethal dose (LD50) of mangosteen extract was 231 mg/kg in mice administered by intraperitoneal injection [37]. The NOAEL for turmeric extract has been evaluated as 3000 mg/kg/day in 28- and 90-day oral toxicity studies in rats [38]. Moreover, a subchronic toxicity study for soybean extract has found that the NOAELs in male and female rats were 707.2 and 751.8 mg/kg/day, respectively [39]. The present work showed no adverse effects in male and female rats with a NOAEL of at least 5470 mg/kg/day SCF by oral administration for 28 days. A MOS value of 100 was used to assess the safety of daily intake for SCF in humans. The results suggest that the safe dose of SCF for human daily intake is 3282 mg (54.7 mg/kg/day for a 60 kg person).

4. Conclusion

In summary, the results of the in vitro bacterial reverse mutation test and chromosome aberration test indicated that SCF treatment did not possess the induction potential for mutagenicity and clastogenicity. There were no observations of body weights, relative organ weights, hematological parameters, serum biochemical analysis, and histopathological abnormalities in SCF-treated groups from the 28-day oral toxicity test in rats. Although some parameters of serum biochemistry at high-dose SCF-treated group in male rats were significantly decreased compared to the control group, these changes were considered incidental, which data were within the normal ranges based on the historical records control data. Moreover, treatment with SCF did not lead to the histological lesions in the high-dose group. Based on these findings, the NOAEL of SCF may be defined as at least 5470 mg/kg/day.

Funding

This study was supported by the grants from the Industrial-Academic Cooperation Project of National Taiwan University, Taiwan (NTU109M130) and the Teh-Tzer Study Group for Human Medical Research Foundation, Taiwan (B1081007).

Ethics approval and consent to participate

All the animal experiments were approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Centre at College of Medicine, National Taiwan University (protocol and approval number:20200209).

CRediT authorship contribution statement

There are no relevant financial or non-financial competing interests to report. Authors:Ting-Yu Chang, Kuo-Cheng Lan, Kuo-Tai Hua, Shing-Hwa Liu.

Authors’ contributions

KTH and SHL designed the study, data interpretation and editing the manuscript. TYC performed the research and wrote the manuscript. KCL and SHL contributed to review the analysis of the data and data interpretation. All authors reviewed the data and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.04.008.
