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Research Article

Assessing systemic, developmental, and reproductive toxicity and estrogenicity of Korean red ginseng extract G1899 in juvenile Sprague-Dawley Rats

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ABSTRACT

Background: Korean red ginseng (KRG) is a product from ginseng roots, which is enriched with ginsenosides and has been utilized for a long time as an adaptogen to alleviate various physiological or disease conditions. While KRG is generally considered safe, conducting a thorough toxicological assessment of the spray-dried powder G1899 during the juvenile period is essential to establish its safety profile. This study aimed to assess the safety of G1899 during the juvenile period using Sprague-Dawley rats.

Methods: Two studies were conducted separately: a juvenile toxicity study and a uterotrophic bioassay. To assess the potential toxicity at systemic, postnatal developmental, and reproductive levels, G1899 was orally gavaged once a day in post-weaning juvenile Sprague-Dawley (SD) rats at 0, 1250, 2500, or 5000 mg/kg/day. Estrogenicity was assessed by orally gavaging G1899 in immature female SD rats at 0, 2500, or 5000 mg/kg/day on postnatal days (PND) 19–21, followed by a uterotrophic bioassay. These studies were conducted in accordance with the Good Laboratory Practice (GLP) regulations and regulatory test guidelines.

Results: Regarding juvenile toxicity, no abnormalities related to the G1899 treatment were observed in any group during the experiment. Moreover, no uterotrophic responses were observed in the dosed female group. Based on these results, the no observed adverse effect level (NOAEL) of G1899 was determined to be at least 5000 mg/kg/day for general systemic function, developmental/reproductive function, and estrogenic activity.

Conclusion: Our results suggest that G1899 is not toxic to juveniles at doses of up to 5000 mg/kg/day.

1. Introduction

Ginseng has been used in East Asian countries for thousands of years as a traditional remedy for enhancing vitality and ameliorating health-related issues [1]. Ginseng species are globally distributed and include *Panax ginseng* Meyer (Korean ginseng), *Panax Japonicus* Meyer (Japanese ginseng), *Panax notoginseng* Burk (Tienchi ginseng), and *Panax quinquefolium* L. (American ginseng) [2]. Among the diverse ginseng species, Korean ginseng is distinguished by its high ginsenoside content, a pivotal bioactive component responsible for its pharmacological effects [3]. In addition, Korean ginseng can undergo an additional transformation process to become what is known as red ginseng (referred to as Hongsam in Korean). This process includes steaming and drying fresh roots without peeling, resulting in the conversion of natural ginsenosides such as Rb (1 and 2), Rc, Rd, Rg1, and Re into Rg (2 and 6), Rh (1 and 4), Rk (1 and 3), Rg (3, 5, 9, and 10), and Rz1. Korean red ginseng (KRG) contains more diverse ginsenosides than fresh ginseng root or white ginseng [4–6]. In light of these discoveries, numerous studies have investigated the efficacy of KRG. These reports indicate that KRG demonstrates adaptogenic effects not only on various physiological systems, including stress, metabolic, endocrine, and immune systems, but also exhibits potential in addressing conditions such as cancer and

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cardiovascular diseases [7–12]. Individuals of all age groups have widely adopted various forms of red ginseng owing to their advantageous pharmacological effects.

From a regulatory or scientific perspective, the safety of ginseng has not been thoroughly scrutinized despite the perception that this traditional herbal remedy is safe for consumption. This lack of research can be attributed to inherent disparities in ginseng product manufacturing processes and the significant variations in content and quality observed across distinct sample batches [13]. Meticulous investigations into the subacute and subchronic toxicity of KRG extract and its developmental toxicity when mixed with other natural products have been conducted under stringent experimental conditions. These studies have provided insights, establishing that oral administration of doses up to 2000 mg/kg/day in rats has no adverse effects [14–16]. Nonetheless, it is imperative to acknowledge that KRG may be consumed by younger populations, whose characteristics differ significantly from those of adult subjects primarily scrutinized in previous toxicity assessments [17]. Unfortunately, there is a significant gap in our understanding of the safety of KRG during developmental phases. Therefore, comprehensive toxicological studies, including those in juveniles, are crucial for a thorough safety assessment.

Consequently, we conducted a comprehensive investigation of the potential toxicological effects of the KRG extract spray-dried powder G1899 during the juvenile developmental phase, primarily focusing on its safety. Notably, G1899 is typically consumed orally as a functional health food after weaning during the juvenile period in humans. Following the International Council for Harmonization (ICH) nonclinical pediatric safety guidelines (S11) [17], we administered G1899 orally to post-weanling (juvenile) Sprague-Dawley (SD) rats. We selected this approach to enable a comprehensive safety assessment covering a spectrum of endpoints, including general toxicity indicators and those related to developmental and reproductive toxicity. Additionally, we evaluated the potential estrogenic properties of G1899 following the Organization for Economic Co-operation and Development (OECD) guidelines, which were established for the testing of chemicals [18]. Our results indicated that G1899 is generally non-toxic and does not affect development and reproduction in juveniles.

2. Materials and methods

2.1. Sample preparation and dose formulation

G1899 was prepared by the Korea Ginseng Corporation (Republic of Korea) as follows. KRG was prepared by steaming 6-year-old *P. ginseng* in compliance with the requirements of the International Organization for Standardization (ISO) 19,610:2017. Both *P. ginseng* and KRG complied with quality control criteria for acceptable levels of pesticides and contaminants. KRG was extracted seven times using 10 vol of distilled water at 85 °C for 10 h and concentrated under vacuum at 60 °C until it reached above 60° brix. Finally, the concentrated extract was dehydrated using a spray-drying method to remove moisture and obtain a light-brown powder (G1899).

Ginsenoside and acidic polysaccharide contents in G1899 were analyzed according to the Notification of the Ministry of Food and Drug Safety for Health Functional Foods (2016–143). 0.3 g of G1899 was dissolved in 20 mL of 70 % methanol and mixed using an ultrasonicator (SD-200H, Sungdong Ultrasonics, Korea). The solution was filtered through a PVDF membrane filter (0.22 μm, φ47 mm, Nylon, Whatman). The filtrate was analyzed for 13 ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2s, Rg3r, Rg3s, Rg5, Rh1, and Rk1) at 203 nm using a UPLC/PDA system (Acquity UPLC System, Waters, USA). The total ginsenoside content of G1899 was >2 %. The polysaccharide content of G1899 was determined by measuring the absorbance at 525 nm (OD) using a microplate reader (SpectraMax M2; Molecular Devices, USA). The polysaccharide content of G1899 was greater than 3 %. The purity of the test substance was 95.8 %, as calculated based on the loss after drying, which accounted for 4.2 % of the total weight. Adjustments for purity were made when formulating G1899, using distilled water (Dae Han Pharm, Republic of Korea) as the vehicle. The stability, homogeneity, and concentration of the dose formulations were analyzed via high-performance liquid chromatography (HPLC) by a validated method using ginsenoside Rb1 as an analytical marker. Dose formulation analyses were conducted twice in the juvenile toxicity study (in the first and last weeks of dosing) and once in the uterotrophic bioassay. As a result, homogeneity was acceptable, as the Coefficient of Variation (CV) was within 5 % of the mean of the top, middle, and bottom results. In addition, the concentration results were acceptable, as the mean concentration was within 15 % of the nominal concentration. The vehicle and dose formulations were administered orally once daily for approximately the same duration. In addition, the administration volume was calculated to be 10 mL/kg based on the most recently measured body weight of the animals.

2.2. Experimental animals

All procedures involving animals were inspected and approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology, in accordance with the Animal Protection Act and the Guide for the Care and Use of Laboratory Animals [19]. Korea Institute of Toxicology has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). In the course of this study, a juvenile toxicity study and a uterotrophic bioassay were conducted separately. Both studies were conducted in full compliance with the OECD Good Laboratory Practice (GLP) regulations [20]. In addition, Specific pathogen-free (SPF) Sprague-Dawley (SD) rats sourced from Orient Bio Incorporation (Republic of Korea) were used in this study. After acclimation, healthy animals were assigned to each study group to ensure comparable mean body weights using the Pristima System (Xybion Medical Systems Corporation, USA). The animals were housed either in stainless steel cages or in polysulfone cages containing autoclaved aspen animal bedding (Doongji Bio, Republic of Korea) throughout the study. In addition, animal enrichment devices (nylon bones) were supplied to each cage for the juvenile toxicity studies. The animal room's environmental conditions were tightly controlled, maintaining a temperature range of 20–26 °C (for the juvenile toxicity study) or 19–25 °C (for the uterotrophic bioassay), with a relative humidity range of 30–70 %, 10–20 air changes per hour ventilation, and 12-h light/12-h dark cycle with light levels between 150 and 300 Lux. All animals were provided a gamma-irradiated standard rodent pellet diet (PMI Nutrition International, USA) *ad libitum* for the juvenile toxicity study and a soy protein-free rodent diet (Envigo, USA) *ad libitum* for the uterotrophic bioassay. Animals had access to filtered and irradiated municipal tap water *ad libitum*. No detectable contaminants were present in the diet, water, bedding, or enrichment devices that could potentially interfere with the outcomes of the study.

2.3. Determining the dose-range

A dose-range-finding study was conducted to assess the potential effects of G1899 and to determine the appropriate dose for a definitive juvenile toxicity study and uterotrophic bioassay. Sixty-four Sprague-Dawley rats (8 animals/sex/group) were orally administered G1899 for 7 weeks starting on postnatal day (PND) 21 at doses of 0, 1250, 2500, or 5000 mg/kg/day. Throughout the study, mortality, clinical signs, body weight, food consumption, clinical pathology (hematology, clinical chemistry, and urinalysis), macroscopic observations, and organ weights were monitored.

2.4. Juvenile toxicity study

The study design of the juvenile toxicity study referred to ICH safety

guideline S11 and a previously published study [21]. Seventy-eight time-mated female were selected (gestational day (GD) 0) and allowed to breed. The size of each litter was adjusted to have four males and four females per litter by randomly removing some pups on PND 4.138 male and 138 female F1 animals were then selected (one or two F1 animals/sex from a litter) on PND 20. F1 animals were subdivided into two cohorts: cohort 1 for general toxicity and cohort 2 for developmental and reproductive toxicity. The dosing initiation age of F1 animals was PND 21 because G1899 can be applied intentionally to humans as early as two years old [22].

2.4.1. General toxicity observations (cohort 1)

One hundred animals (50 males and 50 females, with 15 animals/sex for the vehicle and high-dose groups and 10 animals/sex for the low- and medium-dose groups) were subjected daily to oral gavage administration of G1899 at 0, 1250, 2500, or 5000 mg/kg/day, commencing on PND 21 and continuing until PND 63. Five animals/sex/group in vehicle control and high-dose group were assigned to recovery group, which were investigated for the potential reversibility of toxicity following a 4-week recovery period after completion of dosing.

In-life observations were conducted as follows. General clinical signs including mortality were observed twice daily during the administration period and once daily during recovery period. Detailed clinical signs were observed once a week, preferably on body weight measurement day. Body weight and food consumption were measured twice a week and once a week, respectively. Sexual maturation was observed by recording the completion date of the vaginal opening for females (beginning on PND 26) and preputial separation for males (beginning on PND 36). The body weight was additionally measured on the day sexual maturity was confirmed. All animals in cohort 1 were subjected to clinical pathology examinations. As reported previously, the general hematology (ADVIA2120i, Siemens, Germany), coagulation (ACL, Instrumentation Laboratory, USA), and clinical chemistry (200FR NEO, Toshiba Corporation, Japan) parameters included in this type of repeated-toxicity study were analyzed [23]. For urinalysis, urine color, urine volume, specific gravity, pH, urobilinogen, bilirubin, protein, glucose, ketones, nitrite, leukocytes, and erythrocytes were analyzed (Cobas U411; Roche, Germany). Urine sediment potassium, sodium, and sodium levels were analyzed using a chemistry analyzer (200FR NEO, Toshiba Corporation, Japan). Postmortem examinations, including macroscopic observation, organ weight, and histopathology, were conducted as previously reported [23]. In addition, on day of scheduled sacrifice of cohort 1 animals, the length of left femur was measured to investigate longitudinal growth of bone.

2.4.2. Developmental and reproductive toxicity observations (cohort 2)

A total of 176 animals (88 males and 88 females, with 22 animals/sex/group) were subjected to daily oral gavaging of G1899 at doses of 0, 1250, 2500, or 5000 mg/kg/day: PND 21–98 for males and PND 21- GD 14 for females (approximately 70 consecutive days in total).

As in-life observation for cohort 2 animals were observed in the same manner as in cohort 1. In addition, estrous cycle was examined for its length and regularity by performing a vaginal smear, daily from 2 weeks prior to the mating date. Mating observations were performed as previously described [24]. Briefly, males and females in a 1:2 ratio in the same group (without sibling mating) was mated for two weeks, starting from at a minimum age of 10 weeks. Mating was confirmed every morning by checking for vaginal plugs and/or sperm in vaginal smears.

All animals in cohort 2 were subjected to macroscopic observations, organ weights, cesarean sections (females only), and sperm analyses (males only). All surviving males and females on PND 98 and GD 15, were respectively euthanized using CO_2, and macroscopically observed for abnormalities in their external, cranial, thoracic, and abdominal cavities. The epididymis, seminal vesicles with coagulation glands, prostate, testes, ovaries, and uterus were weighed after macroscopic observation. Cesarean sections were performed as described previously [16]. Briefly, the corpora lutea, implantation sites, resorption (early and late), and live/dead fetuses were observed following caesarean section. Sperm analyses were performed as previously described [23]. Briefly, the motility of caudal epididymal sperm was analyzed using sperm analyzer (HTM-TOX IVOS Hamilton-Thorne Research, USA), morphology and the number of sperms at testis and epididymis were examined microscopically.

2.5. Uterotrophic bioassay

The design of the uterotrophic bioassay adhered to the general principles outlined in OECD test guideline 440 and a previously published study [21]. Sixteen time-mated female rats (GD 0) were allowed to breed. The size of each litter was adjusted to have four males and four females per litter by randomly removing some pups on PND 4. For uterotrophic bioassay, a total of twenty-four F1 females (one or two F1 females from a litter) were selected and grouped into three groups (8 animals/sex/group) on PND 19. G1899 was then administered daily by oral gavage at a dose of 0, 2500, or 5000 mg/kg/day from PND 19 to 21.

General clinical signs including mortality were observed twice daily during administration period and once daily on the terminal sacrifice day. Body weight and food consumption were measured daily throughout the study period. As terminal procedures, all surviving females on PND 22 were euthanized using CO_2 and observed macroscopically for abnormalities in the external, abdominal, thoracic, and cranial cavities, with special attention paid to their reproductive systems. In addition, whether vaginal opening had occurred was observed prior to vaginal dissection. The uterus (wet and blotted uterus) of all animals was weighed at sacrifice. Wet uterus was weighted without drying. Blotted uterus was cut longitudinally and weighted after complete removal of luminal fluid with filter paper. The organ to body weight ratio was calculated from the terminal body weight measured prior to necropsy.

2.6. Statistical analyses

Data were presented as means ± standard deviations. F1 data were evaluated based on the statistical litter units, and data from females identified as non-pregnant were ruled out for analysis. Statistical analyses were carried out using the SAS program version 9.4 (SAS Institute, USA) or Pristima system version 7.4 (Xybion Medical System, USA) [16,25]. $p < 0.05$ or $p < 0.01$ was considered statistically significant.

3. Results

3.1. Juvenile toxicity of G1899

3.1.1. Determining the dose-range

In a dose-range-finding study at doses of 0, 1250, 2500, or 5000 mg/kg/day, no significant changes were observed in clinical signs, clinical chemistry, macroscopic observations, or organ weights related to G1899 treatment. Although there were subtle alterations in body weight gain, food consumption, hematology, and urinalysis, G1899 was considered tolerable up to a dose of 5000 mg/kg/day. Based on the findings of the dose-range study, doses of 0, 1250, 2500, or 5000 mg/kg/day were chosen for the juvenile toxicity study, and doses of 0, 2500, or 5000 mg/kg/day were selected for the uterotrophic bioassay.

3.1.2. Mortality

No G1899-related deaths occurred in the animals in cohorts 1 and 2 throughout the study. The deaths of one male each in the 1250 and 2500 mg/kg/day groups in cohort 2, which occurred before mating and scheduled sacrifice, were considered incidental. This determination was based on the absence of findings during the in-life period and postmortem examination, and mortality was not found to be dose-dependent.

3.1.3. Body weight

Throughout the study period, there were no changes in body weight or body weight gain related to G1899 in any of the groups in cohorts 1 and 2 (Fig. 1). Males and females tended to have lower body weights during the recovery period. However, the decline in body weight during recovery seemed primarily associated with individuals who initially had lower body weights rather than indicating a latent effect. This interpretation is supported by the observation that the body weight of the high-dose recovery group had already decreased on the first day of the recovery period compared to that of the control group. Furthermore, the body weight increment slope was similar between the control and high-dose groups during the recovery period.

3.1.4. Food consumption

No G1899-related change in food consumption were observed in any of the groups in cohorts 1 and 2, as detailed in the supplementary data. Some significant changes were observed, but were not considered to be attributed to G1899, as they were either transient or lacked a dose-response relationship.

3.1.5. Sexual maturity, estrus cycle, and fertility

No G1899-related changes were observed in sexual maturity in cohort 1 and 2 (Table 1), length and regularity of estrus cycle, or fertility indices in cohorts 2 (Table 2).

3.1.6. Clinical pathological parameters

No G1899-related changes were observed in hematology and coagulation, clinical chemistry, or urinalysis in cohort 1, as detailed in the supplementary data. Some significant changes were considered unrelated to G1899 because they were not dose-dependent or were observed only in the recovery group.

3.1.7. Macroscopic observation, organ weights, and microscopic observation

No G1899-related changes were observed macroscopically in both cohorts 1 and 2. Sporadic findings were considered spontaneous, given their low incidence or severity, as detailed in the supplementary data. There were no G1899-related changes in organ weights in cohorts 1 and 2, as detailed in the supplementary data. Significant changes in organ weights were not considered G1899-related, as they were not dose-dependent or observed only at recovery group. The evaluation of systemic tissues by the representative panel revealed no notable histopathological findings associated with G1899 treatment, encompassing the glomerular and tubular structures in the kidney, hepatocyte and lobular structure in the liver, germinal epithelium and germ cell organization in the testes, as well as follicular development in the ovary (Fig. 2).

Table 1
Sexual maturation results of G1899 administered animals in cohort 1 and 2 of juvenile toxicity study.

	G1899 (mg/kg)			
	0	1250	2500	5000
Cohort 1				
Males (number of animals)	15	10	10	15
Preputial Separation (day)	42.8 ± 1.26	43.9 ± 1.73	42.4 ± 2.27	43.9 ± 1.73
Body weight on preputial separation (g)	252.9 ± 22.74	254.8 ± 26.79	244.7 ± 21.68	269.4 ± 21.85
Females (number of animals)	15	10	10	15
Vaginal opening (day)	31.9 ± 1.44	31.6 ± 2.41	32.5 ± .78	31.6 ± .80
Body weight on vaginal opening (g)	127.7 ± 16.86	130.1 ± 20.17	133.2 ± 19.73	127.5 ± 14.97
Cohort 2				
Males (number of animals)	22	22	22	22
Preputial Separation (day)	42.8 ± 1.99	43.5 ± 1.85	42.6 ± 1.14	42.7 ± 1.59
Body weight on preputial separation (g)	255.7 ± 24.43	260.2 ± 22.87	249.2 ± 14.60	251.2 ± 15.99
Females (number of animals)	22	22	22	22
Vaginal opening (day)	32.4 ± 2.20	31.8 ± 1.87	32.9 ± 2.27	32.1 ± 1.46
Body weight on vaginal opening (g)	131.7 ± 15.25	127.1 ± 12.65	133.7 ± 15.04	131.6 ± 13.78

All values are presented as the mean ± standard deviation.

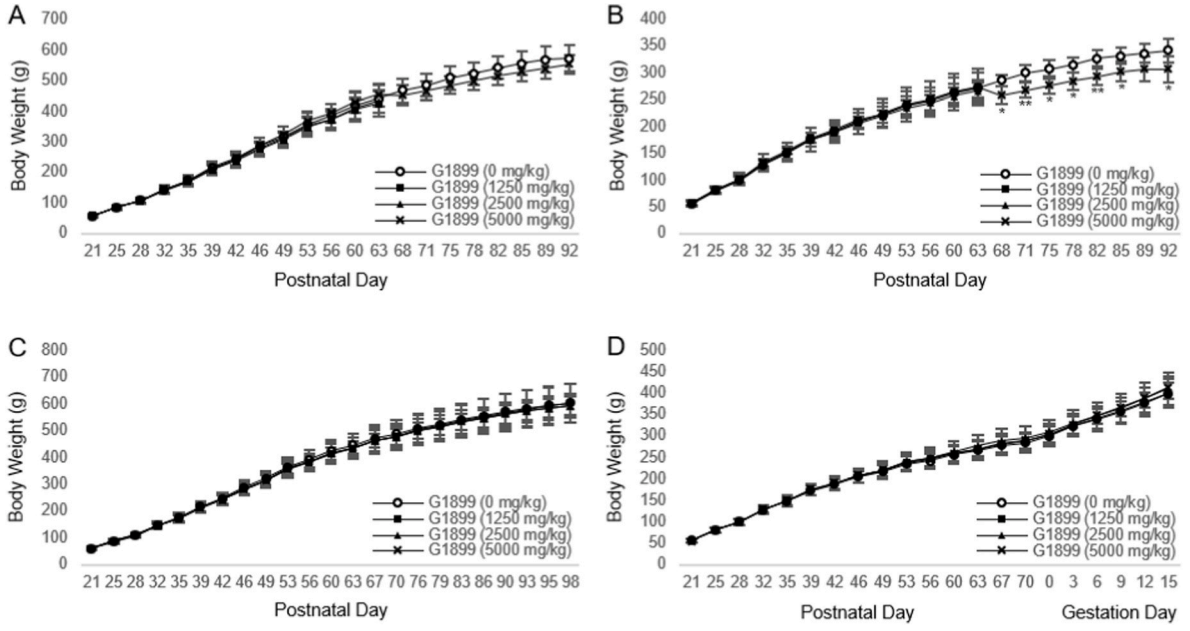

Fig. 1. Average body weight changes G1899 administered animals in the juvenil toxicity study. Two cohort studies were conducted as descrived in Materials and Methods. (A) male rats and (B) female rats in cohort 1, and (C) male rats and (D) female rats in cohort 2. *$p < 0.05$ and **$p < 0.01$ compared with the control group.

Table 2
Estrus cycle examination and fertility-related results of G1899 orally administered animals in cohort 2 of juvenile toxicity study.

	G1899 (mg/kg)			
	0	1250	2500	5000
Number of males	22	21	22	22
Number of females	22	22	22	22
Estrus cycle examinations				
Length (day)				
Mean	4.2	4.2	4.2	4.2
Sdev	0.41	0.40	0.44	0.34
Regularity				
Regular (%)	20 (90.9)	20 (90.9)	20 (90.9)	22 (100.0)
Irregular (%)	2 (9.1)	2 (9.1)	2 (9.1)	0 (0.0)
Fertility indices				
Males				
Mating index[a]	21/22	21/21	22/22	22/22
(%)	(95.5)	(100.0)	(100.0)	(100.0)
Fertility index[b]	21/22	19/21	22/22	22/22
(%)	(95.5)	(90.5)	(100.0)	(100.0)
Fecundity index[c]	21/21	19/21	22/22	22/22
(%)	(100.0)	(90.5)	(100.0)	(100.0)
Females				
Mating index[d]	21/22	22/22	22/22	22/22
(%)	(95.5)	(100.0)	(100.0)	(100.0)
Fertility index[e]	21/22	20/22	22/22	22/22
(%)	(95.5)	(90.9)	(100.0)	(100.0)
Pregnancy index[f]	21/21	20/22	22/22	22/22
(%)	(100.0)	(90.9)	(100.0)	(100.0)
Precoital time (day)				
Mean	2.9	2.7	3.3	2.4
Sdev	2.72	1.13	2.22	1.14

[a] (No. of males with evidence of mating/No. of males paired) × 100.
[b] (No. of males impregnating a female/No. of males paired) × 100.
[c] (No. of males impregnating a female/No. of males with evidence of mating) × 100.
[d] (No. of females with evidence of mating/No. of females paired) × 100.
[e] (No. of pregnant females/No. of females paired) × 100.
[f] (No. of pregnant females/No. of females with evidence of mating) × 100.

3.1.8. *Sperm analysis and caesarean section*

No G1899-related changes in testicular and epididymal sperm count, their morphology and motility were found in all groups in cohort 2 males (Table 3). No G1899-related changes in cesarean section parameters in any of the groups in cohort 2 females (Table 3).

3.2. *Estrogenic activity of G1899*

No G1899-related changes were observed in the mortality, clinical signs, body weight, or food consumption in any immature female rats during the uterotrophic bioassay (Table 4). At terminal observation, no G1899-related changes in the macroscopic findings and uterine weights (both wet and blotted uterine weights) were observed in all groups (Table 4).

4. Discussion

In the juvenile toxicity study, we administered G1899 via oral gavage at 0, 1250, 2500, or 5000 mg/kg/day to juvenile rats from PND 21. Compared to previous toxicity studies conducted with KRG extracts similar to G1899 (5000 mg/kg/day) [15], the dose level that was not used in the general toxicity study was selected as the highest dose level to secure a sufficient safety margin for children consuming dietary supplements containing G1899. While the NOAEL of 5000 mg/kg/day in the juvenile toxicity study may not be directly comparable across studies due to variations in the maximum doses used, our study, which thoroughly considered the safety margin, provides a higher level of safety information compared to previous studies.

Administration period for this study was selected based on the age-dependent development of human and rat organ [17]. The majority of organ, except for nervous system, undergo structural and/or functional maturation pending adulthood, which is corresponded as 18-year old in humans and as 10-week old (PND 70) in rats. Additionally, we continued dosing to investigate mating performance and fertility of treated rats. Therefore, a total administration period of at least 70 days from PND 21 is sufficient for observing potential effects on developing organ systems, especially for reproductive organs.

In this study, no G1899-related changes were found in mortality, clinical signs, body weight, or food consumption during the in-life period in either cohort 1 or 2 animals. Additionally, no abnormalities were observed in these organs in juvenile animals, even at higher doses of up to 5000 mg/kg/day, despite the previously observed effects on the liver, thyroid/parathyroid gland, and spleen in adult male and/or female rats in a 13-week repeated-dose toxicity study [15]. Notably, the significant changes in clinical pathology were also deemed unrelated to G1899, as they lacked dose dependency or were observed only in the recovery group. Moreover, G1899 did not influence the sexual maturation, gonadal function, or reproductive performance of juvenile rats at doses up to 5000 mg/kg/day.

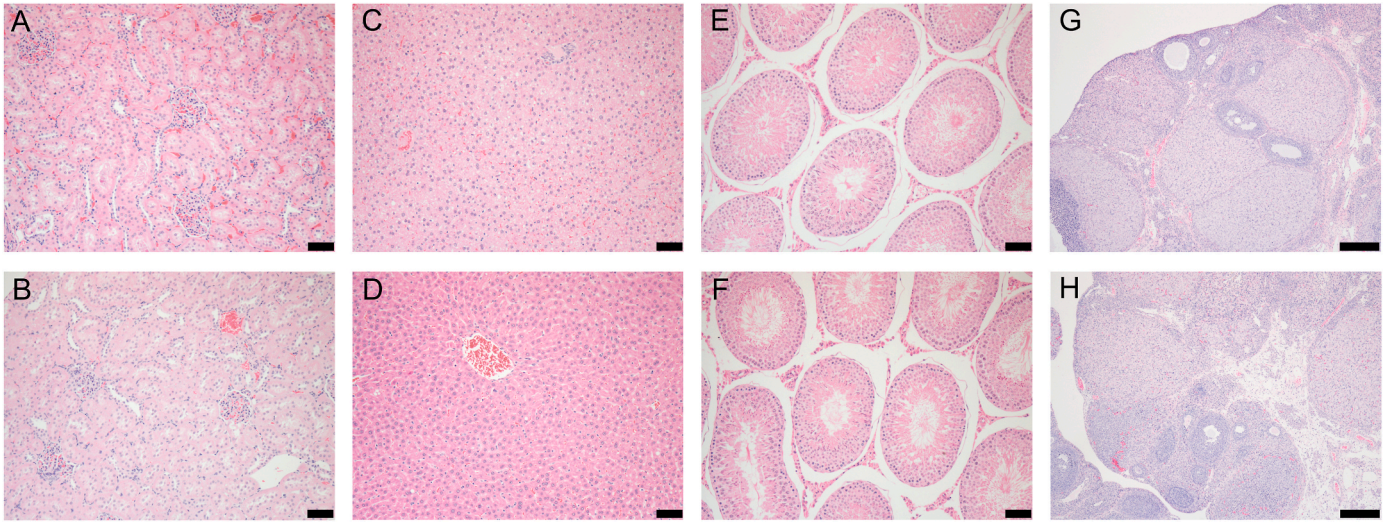

Fig. 2. Hematoxylin & eosin stained tissue slides of the liver (A and B), kidney (C and D) and testis (E and F) in males, and ovary (G and H) in females. Panels A, C, E and G: vehicle control group; Panels B, D, F and H: 5000 mg/kg/day group. Scale bars indicate 50 μm for A to F, and 200 μm for G and H.

Table 3
Sperm analysis and caesarean section results of G1899 administered animals in cohort 2 of the juvenile toxicity study.

	G1899 (mg/kg/day)			
	0	1250	2500	5000
Number of males	22	21	22	22
Sperm number				
Testis (10^6/testis)	129.1 ± 8.15	130.7 ± 6.69	133.5 ± 6.58	137.8 ± 6.99**
Epididymis (10^6/epididymis)	145.3 ± 5.72	151.3 ± 5.19**	147.7 ± 6.15	146.7 ± 7.93
Sperm morphology rowhead				
Normal shape (N)	197.5 ± 1.57	196.4 ± 1.91	197.0 ± 1.48	196.4 ± 1.73
Abnormal shape (N)	2.5 ± 1.57	3.6 ± 1.91	3.0 ± 1.48	3.6 ± 1.73
Morphological abnormality of sperms (%)	1.3 ± .78	1.8 ± 0.95	1.5 ± 0.74	1.8 ± 0.87
Sperm motility rowhead				
MOT (%)	89.32 ± 11.357	90.05 ± 4.399	92.10 ± 3.590	91.73 ± 2.251
VAP (μm/s)	126.6 ± 14.68	124.9 ± 14.71	123.0 ± 10.57	123.2 ± 12.58
VSL (μm/s)	84.5 ± 11.37	82.4 ± 10.71	81.0 ± 7.40	83.3 ± 9.44
VCL (μm/s)	255.6 ± 26.90	255.9 ± 24.75	250.4 ± 22.39	245.3 ± 22.92
ALH (μm)	17.5 ± 1.81	17.2 ± 2.08	17.7 ± 1.53	17.1 ± 1.96
BCF (Hz)	19.2 ± 2.04	18.8 ± 2.14	18.2 ± 1.31	19.0 ± 2.23
STR (%)	68.2 ± 5.79	67.7 ± 5.17	67.3 ± 4.48	39.2 ± 4.92
LIN (%)	33.8 ± 3.43	33.1 ± 2.99	33.5 ± 3.27	35.1 ± 3.98
Number of pregnant females	21	20	21	22
Corpora lutea (N)	16.6 ± 1.80	16.9 ± 2.54	16.9 ± 2.28	17.1 ± 1.60
Implantation (N)	15.8 ± 1.97	16.1 ± 2.67	15.8 ± 3.77	16.8 ± 1.69
Early resorption (N)	0.4 ± .060	0.9 ± 1.12	0.9 ± 0.94	0.6 ± 0.73
Late resorption (N)	0.1 ± 0.44	0.2 ± 0.70	0.1 ± 0.48	0.0 ± 0.21
Dead fetus (N)	0.0 ± 0.00	0.1 ± 0.22	0.0 ± 0.00	0.0 ± 0.00
Fetal death[a] (N)	0.5 ± 0.81	1.2 ± 1.42	1.0 ± 0.97	0.7 ± 0.72
Live fetus (N)	15.2 ± 1.97	15.0 ± 3.12	14.7 ± 3.91	16.1 ± 1.95
Pre-implantation loss[b] (%)	4.5 ± 9.35	4.5 ± 7.57	7.5 ± 18.62	1.9 ± 3.36
Post-implantation loss[c] (%)	3.2 ± 5.05	7.5 ± 9.14	8.6 ± 11.38	4.2 ± 4.60

Abbreviations: MOT, percentage of motile sperm; VAP, average velocity of the smoothed cell path; VSL, average velocity measured in a straight line from the beginning to the end of the track; VCL, the average velocity measured over the actual point-to-point track followed by the cell; ALH, the mean width of the head oscillation as the sperm cells swim; BCF, frequency of sperm head crossing the average path in either direction; STR, average value of the ratio VSL/VAP; LIN, average value of the ratio VSL/VCL.
All values are presented as the mean ± standard deviation, $^{**}p < 0.01$ compared with the control group.
[a] No. of resorptions/litter + No. of dead fetus/litter.
[b] [(No. of corpora lutea/litter – No. of implantation/litter)/No. of corpora lutea/litter] × 100.
[c] [(No. of implantation/litter – No. of live fetus/litter)/No. of implantation/litter] × 100.

Table 4
Body weight and uterine weight of G1899 administered animals in the uterotrophic bioassay.

		G1899 (mg/kg/day)		
		0	2500	5000
Number of females		8	8	7
Body weight gain during the study period (g)		12.7 ± 1.45	9.6 ± 8.07	12.4 ± 2.28
Food consumption during the study period (g/animal/day)		7.1 ± 0.33	6.5 ± 1.19	6.7 ± 0.71
Uterine weight				
Wet uterine weight	Absolute (g)	0.0345 ± 0.00194	0.0338 ± 0.00590	0.0317 ± 0.00489
	Relative (%)	0.0576 ± 0.00550	0.0594 ± 0.00632	0.0535 ± 0.00998
Blotted uterine weight	Absolute (g)	0.0320 ± 0.00214	0.0314 ± 0.00593	0.0290 ± 0.00489
	Relative (%)	0.0533 ± .00547	0.0550 ± 0.00596	0.0490 ± 0.00936

All values are presented as the mean ± standard deviation.

Although ginseng is commonly perceived as safe owing to its natural origins and extensive historical use, it is imperative to conduct comprehensive toxicological investigations to establish its safety profiles. Several clinical trials and comprehensive case report reviews involving products containing ginseng have yielded robust conclusions, indicating that *P. ginseng* is generally well-tolerated, and any adverse effects, when present, tend to be mild and reversible [26]. In subacute, subchronic, and developmental toxicity studies involving KRG extract conducted in animals, no adverse effects were detected, even at the highest doses of 2000 mg/kg/day [14–16].

However, ensuring safety during the juvenile phase is challenging. This period is characterized by rapid growth, as well as distinct physiological difference from adults due to development of organ systems after birth. Consequently, juveniles are generally more vulnerable and sensitive to chemical exposures [27,28]. Moreover, although safety assessments can often be conducted through clinical trials involving adults, conducting such trials on juvenile subjects raises ethical concerns. To address these challenges, juvenile animal studies have assumed a pivotal role in the rigorous and comprehensive safety assessment during this critical developmental phase [29]. To our knowledge, there is a shortage of published studies investigating the toxicity of KRG extract, including assessments during the juvenile period. Hence, we conducted a juvenile toxicity study and a uterotrophic bioassay with G1899, a KRG extract spray-dried powder prepared using a standardized and reproducible process, respectively. Additionally, considering regulatory utilization, these studies complied with GLP regulations and prescribed test guidelines [17,18,20].

The primary focus of the juvenile toxicity study was to investigate potential effects on the postnatal development in rats. From this perspective, we thoroughly examined reproduction in the cohort 2, incorporating endpoints such as sexual maturation and gonadal functions, aligned with the parameters of fertility and early embryonic development study. On the other hands, a comprehensive evaluation of the entire female reproductive cycle, spanning organogenesis to parturition, was not conducted in our study. Although no adverse effects related to G1899 were observed in our study, assessing the birth rate could provide additional insights into reproductive outcomes. Integrating additional studies on the embryo-fetal and pre-/postnatal development study for G1899 could be considered to further strengthen our conclusion.

Phytoestrogens, naturally present in plants, may act directly on estrogen receptors due to their structural similarity to estrogen [31]. Importantly, animal studies have shown phytoestrogens to affect the

hypothalamus-pituitary-gonad (HPG) axis, which is closely linked to sexual development, causing altered timing of puberty and the estrous cycle [32]. To evaluate the potential estrogenicity of G1899, we used an immature ovariectomized myometrium assay (immature method) based on the protocol used in a GLP-compliant OECD validation study. This method is rigorous and appropriate for our purposes because it has similar sensitivity and reproducibility as uterotrophic assay using the ovariectomized adult female rats. Moreover, a broader range of investigations is possible because the HPG axis remains intact, enabling it to respond to test items that interact with the hypothalamus, pituitary gland, or gonads, as well as directly interact with estrogen receptors in the uterus [18]. In immature female rats exposed to G1899, even at a dose of 5000 mg/kg/day, no changes were observed in both wet and blotted uterine weights, nor were any findings associated with sexual maturation identified. These findings indicate that G1899 lacks estrogenic action and simultaneously does not stimulate the HPG axis directly or indirectly. Consequently, it implies that G1899 is unlikely to impact normal sexual development during juvenile periods. These findings are consistent with previous studies showing that KRG has negligible estrogen-like effects in ovariectomized mice (200–800 mg/kg/day) without steroidogenic activity (estrogen and testosterone production) [33]. On the contrary, some researchers have cautiously proposed that ginseng extract and KRG, especially ginsenosides such as Rb1, Rg1, and Rg3, could be considered potential candidates for phytoestrogens based on the observed induction of estrogen receptor (ER) activity *in vitro* [34, 35]. In the same context, KRG has been evaluated in clinical trials to significantly improve symptoms caused by estrogen deficiency in postmenopausal women [36, 37, 38]. However, these improvements are unlikely to be attributed to direct activation of estrogen receptors by KRG, as the possibility that they are mediated through other signaling pathways has not been ruled out. Based on previous uterotrophic bioassay studies on KRG and our results with G1899, the whole extract of KRG does not seem to exhibit estrogen-like activity *in vivo*; however, further investigation into the gap between the *in vitro* and *in vivo* estrogenic activity of KRG is required.

As a dietary supplement, the daily intake of KRG for Korean adults is limited to a range of 2.4–80 mg of ginsenosides (sum of Rg1, Rb1, and Rg3) [39]. A recent study demonstrated the safety and tolerability of 3 g daily intake of KRG extract in healthy adults over a 24-weeks period [40]. However, specific guidelines for daily intake of KRG for juveniles are yet to be established. A single clinical trial reported that children aged 6–12 years who were administered 3 mg of KRG alongside 500 mg of omega-3 for 12 weeks, showed improvements in symptoms related to Attention Deficit Hyperactivity Disorder (ADHD) and cognitive functions without any observed adverse effects [41]. Based on comprehensive toxicity study, we can calculate that juvenile rats can be humans aged 2–18 years, which is supported by the correlation between rats and humans age. Additionally, a dose of 5000 mg/kg/day of G1899 in rats is equivalent to a dose of 1.2 g in 20 kg child (approximately 7–8 years of age) when employing body surface area-based animal-to-human equivalent dose [22, 42, 43]. While the need for specific guidelines for the daily intake of KRG as a dietary supplement for children is recognized, such guidelines are currently lacking. Given this gap, our findings emphasize providing minimal toxicity information in the juvenile period as well as fundamental data for future clinical applications of G1899.

In conclusion, the safety of G1899 is supported by this juvenile toxicity study and the uterotrophic bioassay, which showed no adverse outcomes induced by G1899 in general systemic function and developmental/reproductive function for concentrations up to 5000 mg/kg/day. Furthermore, G1899 did possess no HPG axis-related estrogen-like activity. Therefore, NOAEL of G1899 was considered 5000 mg/kg/day for general systemic, developmental, reproductive function, and estrogenic activity during the experimental period. These results may provide useful information for further use of G1899 in clinical settings.

Declaration of competing interest

Byung-Cheol Han and Eunji Kim are current employees of the Korea Ginseng Corporation that are responsible for the research and development of G1899. The others declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.01.002.

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