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Research Article

Developmental and reproductive toxicity assessment in rats with KGC-HJ3, Korean Red Ginseng with *Angelica gigas* and *Deer antlers*

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ABSTRACT

Background: Korean Red Ginseng has been widely used in traditional oriental medicine for a prolonged period, and its pharmacological effects have been extensively investigated. In addition, *Angelica gigas* and *deer antlers* were also used as a tonic medicine with Korean Red Ginseng as the oriental herbal therapy.
Methods: This study was conducted to evaluate the potential toxicological effect of KGC-HJ3, Korean Red Ginseng with *angelica gigas* and *deer antlers*, on reproductive and developmental functions including fertility, early embryonic development, maternal function, and embryo-fetal development. KGC-HJ3 was administered by oral gavage to Sprague–Dawley rats (22 animals per sex per group) at dose levels of 0 mg/kg (control), 500 mg/kg, 1000 mg/kg, and 2000 mg/kg to evaluate the potential toxicological effect on fertility and early embryonic development. In addition, KGC-HJ3 was also administered by oral gavage to mating-proven Sprague–Dawley rats (22 females per group) during the major organogenesis period at dose levels of 0 mg/kg (control), 500 mg/kg, 1000 mg/kg, and 2000 mg/kg to evaluate the potential toxicological effect on maternal function and embryo-fetal development.
Results and conclusion: No test item–related changes in parameters for fertility, early embryonic development, maternal function, and embryo-fetal development were observed during the study period. On the basis of these results, it was concluded that KGC-HJ3 did not have toxicological potential on developmental and reproductive functions. Therefore, no observed adverse effect levels of KGC-HJ3 for fertility, early embryonic development, maternal function, and embryo-fetal development is considered to be at least 2000 mg/kg/day.

1. Introduction

Korean Red Ginseng is produced with repeated drying and streaming process of raw ginseng to intensify its pharmacological effects. It has been widely used as cosmetics, nutritional supplements, and favorite food including tea and candy as well as medicinal formulations. The beneficial effects of Korean Red Ginseng including antihypertensive, antistress, anticancer, antidiabetes, and antinephrotoxic properties are already investigated in previous studies [1–5].

Angelica gigas and *deer antlers* have been also used as a tonic medicine with Korean Red Ginseng. Pharmacological effects of *Angelica gigas* such as antihypercholesterolemia, antiinflammatory, and antiplatelet aggregation were investigated [6–8]. In addition, *deer antlers* have been investigated its beneficial effect in immune system, physical strength, and sexual function [9,10].

Korean Red Ginseng, *Angelica gigas*, and *deer antlers* have been widely used as a traditional herbal medicine in East Asian countries for many years, but its toxicological potentials have not been entirely investigated. Although individual components including Korean Red Ginseng, *Angelica gigas*, and *deer antlers* have been recognized as relatively nontoxic based on the long history of traditional use in human, there were no sufficient toxicological investigations for its potential adverse effects on human health. In

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particular, there was no published information of the safety assessment in reproductive and developmental aspects for *Angelica gigas* and *deer antlers*, and some ginsenosides Rb1 and Re were recently reported as embryotoxic in the whole embryo culture model [11,12]. In addition, these components have been mainly exposed to human as a mixture form, but its potential toxicological effect after the mixture exposure of these components was not investigated.

For this reason, we investigated the potential effect of KGC-HJ3, Korean Red Ginseng with *Angelica gigas* and *deer antlers*, on developmental and reproductive functions. KGC-HJ3 was administered by oral gavage at dose levels of 0 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg to evaluate the potential effect on fertility and early embryonic development in Sprague–Dawley (SD) rats. In addition, KGC-HJ3 was also administered by oral gavage at dose levels of 0 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg to evaluate the maternal function and embryo-fetal development in SD rats.

2. Materials and methods

2.1. Test item and dose preparation

The test item, KGC-HJ3, Korean Red Ginseng with *angelica gigas* and *deer antlers*, was supplied by the Korea Ginseng Corp. (Republic of Korea). The extract powder of three herbs was mixed in a ratio of Korean Red Ginseng: deer antlers: angelica gigas, 58.4%: 34.1%: 7.5%. The three herbs were extracted with an eightfold volume of water for 8 h at 95°C three times. After cooling for 8 h at 15°C, the extract was centrifuged and filtered through 5 μm pore size. Concentration of the extract was performed at 58°C under 680 mmHg, and the concentrate was finally freeze-dried. Individual ginsenosides composition in KGC-HJ3 was analyzed by high performance liquid chromatography (HPLC) using C18 column. The results of HPLC chromatogram and its concentration are reported in Fig. 1.

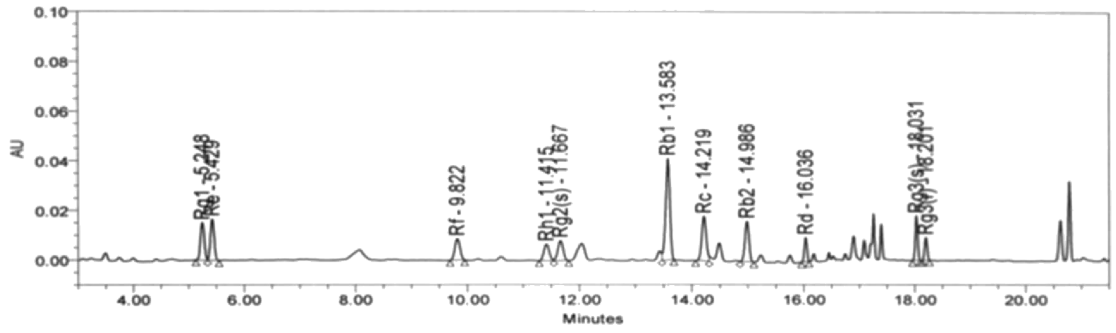
The test item was suspended in distilled water (Daehan Pharm., Republic of Korea). The mixture of the test item with vehicle was prepared every day during the dosing period, and it was administered by oral gavage at approximately the same time each day. All animals were dosed at a volume of 10 mL/kg based on the most recently recorded body weight, and dosing formulation was continuously stirred by a magnetic stirrer during the administration procedure.

2.2. Animals and maintenance

Specific pathogen-free SD rats were purchased from Orient Bio Inc. (Republic of Korea). Animals were used after one week of acclimation period. Grouping was carried out using Pristima Systems (Xybion Medical Systems Co., USA), so that animals in each group have a similar body weight distribution. Animals were housed in a stainless-steel cage (255W × 465L × 200H mm) or polycarbonate cage (240W × 390L × 180H mm) with autoclaved aspen animal bedding material (Bio Lab, Republic of Korea). The animal room was maintained with a temperature range of 17–23°C, relative humidity range of 30–70%, ventilation range of 10–20 air change per hour, and a 12-hour light/12-hour dark cycle with 150–300 Lux and ultraviolet irradiation; filtered tap water was proved *ad libitum*, and standard rodent pellets (PMI Nutrition International, USA) which was irradiated by gamma ray were given *ad libitum*. No hazardous level of contaminants was detected in the water, food, and bedding materials that might have affected the results of this study.

The experiments were reviewed by the Institutional Animal Care and Use Committee of Korea Institute of Toxicology. Korea Institute of Toxicology received full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International since 1998. Animals used on this study were cared for in accordance with the principles outlined in the "Guide for the Care and Use of Laboratory Animals" [13]. In addition, these studies were conducted in accordance with the domestic and international Good Laboratory Practice regulations [14,15] and regulatory test guidelines [16,17].

A



B

Ginsenosides Concentration (mg/g)

Rg1	Rb1	Rg3s	Re	Rc	Rb2	Rd	Rf	Rh1	Rg2	Rg3r
1.66	6.92	1.13	1.85	2.97	2.62	0.81	1.06	0.77	1.04	0.62

Fig. 1. HPLC analysis results for ginsenosides composition and profiles of KGC-HJ3. (A) HPLC chromatograms. (B) Ginsenosides profile and concentration.

2.3. Experimental design for fertility and early embryonic developmental toxicity

Four groups of 22 males (6 weeks old at first dosing) and 22 females (8 weeks old at first dosing) SD rats were daily administered at dose levels of 0 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg to evaluate the potential toxicity of KGC-HJ3 on fertility and early embryonic development. The dose levels in this study were selected based on the previous 4-week repeated-toxicity study in SD rats where KGC-HJ3 at 2000 mg/kg was nontoxic during the study period (data not shown). The KGC-HJ3 was administered daily to male rats beginning 28 days before cohabitation and continuing through the day before sacrifice (total 48 days) and to female rats beginning 14 days before cohabitation and continuing through gestation day (GD) 6.

For in-life observations, mortality and clinical signs were examined at least twice a day to detect any visible abnormalities and to observe the general health of the animals. Body weights were measured during the premating, cohabitation, postmating period (twice a week), and gestation period (GD 0, 3, 6, 9, 12, and 15), and food consumption was also measured in the same interval, except cohabitation period. A vaginal smear was taken daily for each female for two weeks during the premating period, and regularity and length of the estrus cycle were evaluated based on the microscopic cellular stage assessment. After premating period, one female was cohabitated with one male for at least 2 weeks. The first 24-hour period after mating was designated as day 0 of gestation, if sperms or vaginal plugs were detected, and successful copulation was decided by identifying implantation sites in uterus at Caesarean section. Based on the results of mating procedure, fertility-related indices and precoital time were calculated.

For terminal procedures, all surviving males were humanely sacrificed by CO_2 overdose inhalation after the confirmation of pregnancy results, and all surviving females were subjected to

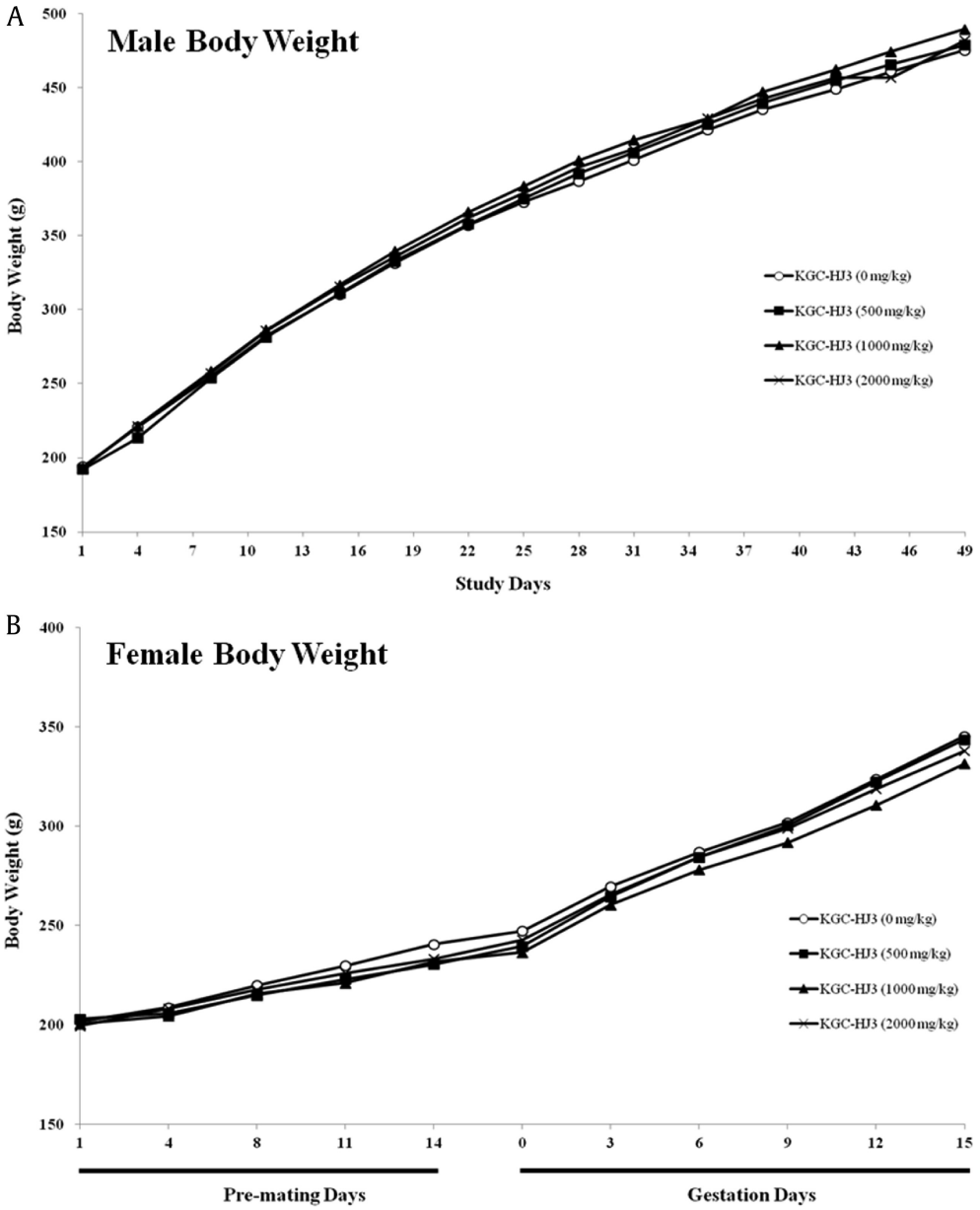


Fig. 2. Mean body weight changes of rats in fertility and early embryonic study. (A) Male rats. (B) Female rats. Four groups were treated with KGC-HJ3 at dose levels 0 (○), 500 (■), 1000(▲), and 2000 (×) mg/kg. The rats treated with KGC-HJ3 showed no significant difference compared with the control.

Caesarean section on GD 15. A complete necropsy with macroscopic findings, major organ weights (brain, heart, pituitary gland, thymus, adrenal glands, lung, liver, spleen, kidneys, testes, epididymides, seminal vesicles with coagulation gland, prostate, ovaries, and uterus with cervix), and histopathological examinations of reproductive organs (testes, prostate, epididymides, seminal vesicles with coagulation gland, vagina, ovaries, and uterus with cervix) was conducted for males and females. In addition, sperm analysis for motility, number, and morphology was conducted for males, and Caesarean section–related parameters were calculated for females.

2.4. Experimental design for embryo-fetal developmental toxicity

Four groups of 22 mating-proven females were administered at dose levels of 0 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg to evaluate the potential toxicity of KGC-HJ3 on maternal function and embryo-fetal development including teratogenic potential. The dose levels were selected based on the previous dose range–finding embryo-fetal development study in SD rats where KGC-HJ3 at 2000 mg/kg was nontoxic during the study period (data not shown). The KGC-HJ3 was administered by oral gavage once daily during the major organogenesis period, from GD 6 to 17.

For in-life observations, mortality and clinical signs were examined at least twice a day to detect any visible abnormalities and to observe the general health of the animals. Body weight and food consumption were measured on GD 0, 6, 9, 12, 15, 17, and 21. On GD 21, all surviving females were humanely sacrificed by CO_2 overdose inhalation and then examined carefully for external, abdominal, thoracic, and cranial cavities abnormalities. For apparently nonpregnant animals, implantation sites were checked according to the ammonium sulfide staining method [18]. Major organ weights (liver, kidneys, lung, spleen, heart, brain, pituitary gland, adrenal glands, and ovaries) were measured during the necropsy procedure. Gravid uterus were weighed and retrieved for Caesarean section, and then, the live/dead fetuses, resorptions (early or late), implantation sites, and corpora lutea were counted. All live fetuses and placenta retrieved from gravid uterus were weighed. Placentas were immediately examined macroscopically, and live fetuses were examined external abnormalities and sexed. Approximately half of the live fetuses were fixed with Bouin's solution for visceral examination. Modified Wilson's method [19] for head, Nishimura method [20] for thorax, and modified Staples method [21] for abdomen were used to examine the visceral abnormalities. The other fetuses were used for evaluation of skeletal abnormalities after staining and examined according to modified Dawson's method [22]. Fetal morphological abnormalities were

Table 1
Selected organ weights, histopathological examination, and sperm analysis of males treated with KGC-HJ3 in fertility and early embryonic study

Parameter		KGC-HJ3 (mg/kg)			
		0	500	1000	2000
Animal (N)		22	22	22	22
Organ weights					
Terminal body weight (TBW)		468.0 ± 31.1[1)]	472.5 ± 39.3	481.5 ± 37.3	464.0 ± 31.2
Liver (g)		17.00 ± 1.78	17.00 ± 1.67	18.00 ± 2.40	16.49 ± 1.74
Per terminal body weight (%)		3.63 ± 0.22	3.60 ± 0.16	3.73 ± 0.30	3.55 ± 0.21
Kidneys (g)		3.71 ± 0.33	3.61 ± 0.35	3.86 ± 0.37	3.76 ± 0.32
Per terminal body weight (%)		0.79 ± 0.06	0.76 ± 0.05	0.80 ± 0.06	0.81 ± 0.06
Adrenal gland (g)		0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.06 ± 0.01
Per terminal body weight (%)		0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Brain (g)		2.07 ± 0.30	1.98 ± 0.11	2.04 ± 0.12	2.00 ± 0.14
Per terminal body weight (%)		0.44 ± 0.08	0.42 ± 0.03	0.43 ± 0.04	0.43 ± 0.04
Prostate (g)		0.63 ± 0.19	0.55 ± 0.13	0.57 ± 0.13	0.62 ± 0.10
Per terminal body weight (%)		0.13 ± 0.04	0.12 ± 0.02	0.12 ± 0.03	0.13 ± 0.02
Thymus (g)		0.47 ± 0.09	0.46 ± 0.08	0.52 ± 0.09	0.52 ± 0.09
Per terminal body weight (%)		0.10 ± 0.02	0.10 ± 0.01	0.11 ± 0.02	0.11 ± 0.02
Seminal vesicles with coagulating gland (g)		1.94 ± 0.33	1.81 ± 0.18*	1.98 ± 0.24	1.89 ± 0.17
Per terminal body weight (%)		0.42 ± 0.08	0.39 ± 0.06	0.41 ± 0.06	0.41 ± 0.04
Right testis (g)		1.56 ± 0.10	1.59 ± 0.12	1.66 ± 0.12*	1.63 ± 0.11
Per terminal body weight (%)		0.33 ± 0.03	0.34 ± 0.04	0.35 ± 0.03	0.35 ± 0.03
Left testis (g)		1.55 ± 0.10	1.59 ± 0.11	1.65 ± 0.08**	1.61 ± 0.12
Per terminal body weight (%)		0.33 ± 0.03	0.34 ± 0.03	0.34 ± 0.03	0.35 ± 0.03
Right epididymis (g)		0.63 ± 0.07	0.63 ± 0.06	0.67 ± 0.05	0.65 ± 0.04
Per terminal body weight (%)		0.13 ± 0.02	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
Left epididymis (g)		0.62 ± 0.05	0.62 ± 0.06	0.64 ± 0.04	0.63 ± 0.04
Per terminal body weight (%)		0.13 ± 0.01	0.13 ± 0.02	0.13 ± 0.01	0.14 ± 0.01
Histopathological examination					
Epididymides					
Infiltration, mononuclear cell	Grade 1	1	–	–	0
Prostate gland					
Infiltration, mononuclear cell	Grade 1	2	–	–	5
	Grade 2	3	–	–	5
	Grade 3	3	–	–	2
Seminal vesicles with coagulating gland					
Infiltration, mononuclear cell	Grade 1	1	–	–	0
Sperm analysis					
Motility (%)		92.4 ± 3.17	90.0 ± 3.65	90.2 ± 4.47	87.9 ± 6.82
Morphological abnormality of sperms (%)		1.7 ± 0.85	2.5 ± 1.97	2.1 ± 1.81	2.6 ± 2.33
No. of sperms in testis (10^6/testis)		141.0 ± 9.81	135.3 ± 9.24	135.8 ± 7.96	138.6 ± 8.40
No. of sperms in epididymides (106/epididymis)		78.2 ± 6.61	73.1 ± 6.35	66.1 ± 5.02**	75.2 ± 8.77

–, not examined
[1)] Values are presented as mean ± S.D.

Table 2
Selected organ weights, histopathological examination, estrus cycle change, and fertility data of females treated with KGC-HJ3 in fertility and early embryonic study

Parameter	KGC-HJ3 (mg/kg)			
	0	500	1000	2000
Animal (N)	22	21	22	22
Organ weights				
Terminal body weight (TBW)	339.3 ± 23.4[1)]	338.4 ± 21.8	323.1 ± 19.8	331.99 ± 20.5
Liver (g)	16.03 ± 1.53	15.74 ± 1.11	15.40 ± 1.19	15.38 ± 1.14
Per terminal body weight (%)	4.72 ± 0.28	4.66 ± 0.25	4.77 ± 0.21	4.63 ± 0.24
Kidneys (g)	2.61 ± 0.24	2.55 ± 0.27	2.53 ± 0.16	2.47 ± 0.20
Per terminal body weight (%)	0.77 ± 0.05	0.75 ± 0.07	0.79 ± 0.06	0.75 ± 0.05
Adrenal gland (g)	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
Per terminal body weight (%)	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Brain (g)	1.86 ± 0.09	1.88 ± 0.09	1.87 ± 0.08	1.86 ± 0.09
per terminal body weight (%)	0.55 ± 0.05	0.56 ± 0.04	0.58 ± 0.03	0.56 ± 0.04
Thymus (g)	0.54 ± 0.09	0.51 ± 0.10	0.51 ± 0.14	0.51 ± 0.08
Per terminal body weight (%)	0.16 ± 0.02	0.15 ± 0.03	0.16 ± 0.04	0.15 ± 0.02
Uterus with cervix (g)	18.76 ± 1.73	17.55 ± 2.90	17.66 ± 2.04	17.99 ± 3.50
Per terminal body weight (%)	5.53 ± 0.41	5.17 ± 0.76	5.47 ± 0.54	5.43 ± 1.00
Right ovary (g)	0.06 ± 0.02	0.07 ± 0.01	0.06 ± 0.02	0.06 ± 0.01
Per terminal body weight (%)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
Left ovary (g)	0.07 ± 0.02	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.01
Per terminal body weight (%)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Animal (N)	22	–	–	22
Histopathological examination				
Ovaries				
Cyst (s)	0	–	–	1
Animal (N)	**22**	**22**	**22**	**22**
Estrus cycle change				
Regular cycle (N)	22	21	22	22
Irregular cycle (N)	0	1	0	0
Mean length (days)	4.2 ± 0.36	4.0 ± 0.11	4.1 ± 0.23	4.1 ± 0.29
Animal (N)	**22**	**22**	**22**	**22**
Fertility data				
Mating index[2)] (%)	22/22 (100)	21/22 (95.5)	22/22 (100)	22/22 (100)
Fertility index[3)] (%)	22/22 (100)	21/22 (95.5)	22/22 (100)	22/22 (100)
Pregnancy index[4)] (%)	22/22 (100)	21/21(100)	22/22 (100)	22/22 (100)

–, not examined
1) Values are presented as mean ± standard deviation.
2) No. of animals with successful copulation/No. of animals with mated.
3) No. of animals with pregnancy/No. of animals with mated.
4) No. of animals with pregnancy/No. of animals with successful copulation.

classified as malformations or variations according to the severity of abnormalities based on institutional criteria. The terminology for fetal morphological abnormalities was used as suggested in an internationally developed glossary of terms for structural developmental abnormalities in common laboratory mammals [23].

2.5. Statistical analysis

Statistical analyses for comparisons of the various dose groups with the vehicle control group were conducted using Pristima System (Xybion Medical Systems Co.) or Statistical Analysis Systems (SAS/STAT User's Guide Version 9.2, USA). Multiple comparison tests for different dose groups were conducted. Continuous data were examined the variance of homogeneity using the Bartlett's test. Homogeneous data were analyzed using the analysis of variance, and the significance of intergroup differences was analyzed using Dunnett's test. Heterogeneous data were analyzed using Kruskal–Wallis test, and the significance of intergroup differences between the control and treated groups was assessed using Dunn's rank sum test. Categorical data were

Table 3
Caesarean section data of females treated with KGC-HJ3 in fertility and early embryonic study

Parameter	KGC-HJ3 (mg/kg)			
	0	500	1000	2000
Animal (N)	22	21	22	22
Corpora lutea (N)	16.7 ± 2.15[1)]	15.9 ± 1.84	16.0 ± 2.63	16.4 ± 2.24
Implantation (N)	15.9 ± 1.48	15.4 ± 2.16	15.0 ± 1.89	15.4 ± 2.77
Early resorptions (N)	0.5 ± 0.74	0.4 ± 0.75	0.4 ± 0.73	0.6 ± 0.91
Late resorptions (N)	0.3 ± 0.46	0.3 ± 0.90	0.3 ± 0.55	0.4 ± 0.79
Dead fetuses (N)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Fetal death (resorptions + dead fetuses)	0.8 ± 0.69	0.7 ± 1.06	0.6 ± 0.85	1.0 ± 1.13
Live fetuses (N)	15.1 ± 1.42	14.7 ± 2.22	14.3 ± 1.91	14.5 ± 2.97
Preimplantation loss (%)[2)]	4.1 ± 5.80	3.2 ± 5.78	5.9 ± 8.46	6.2 ± 12.21
Postimplantation loss (%)[3)]	4.8 ± 4.29	4.5 ± 6.92	4.2 ± 5.50	6.2 ± 7.59

1) Values are presented as mean ± standard deviation.
2) [(No. of corpora lutea − No. of implantation)/No. of corpora lutea] × 100.
3) [(No. of implantation − No. of live fetuses)/No. of implantation] × 100.

analyzed by χ^2 test followed by the Fisher's exact test or ranked Kruskal–Wallis (H). One-way analysis of covariance was used to analyze fetal and placental weight data. Numerical data obtained during the conduct of the study were subjected to calculation of group means and standard deviations. Litter data was statistically evaluated using the statistical unit as a litter. Data were considered to be significant when $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Fertility and early embryonic development study

There was no treatment-related mortality and abnormal clinical signs in fertility and early embryonic development study. In body weight (Fig. 2) and food consumption (data not shown) observation, there were no treatment-related changes in any of the treatment groups. At terminal observation, there were no treatment-related macroscopic findings (data not shown) and organ weights (Table 1 and Table 2). In organ weight, a statistically significant decreased seminal vesicle in males at 500 mg/kg and a statistically significant increased testis in males at 1000 mg/kg were observed, which were not considered treatment related, given the lack of dose dependency and no related change in other parameters.

In fertility and early embryonic development, there were no treatment-related changes, including estrus cycle, precoital time, fertility indices, sperm analysis, Caeasarean section, and histopathological examinations of reproductive organs (Table 1, Table 2 and Table 3). In sperm analysis, a statistically significant decrease in the number of sperm in testis at 1000 mg/kg was observed, which

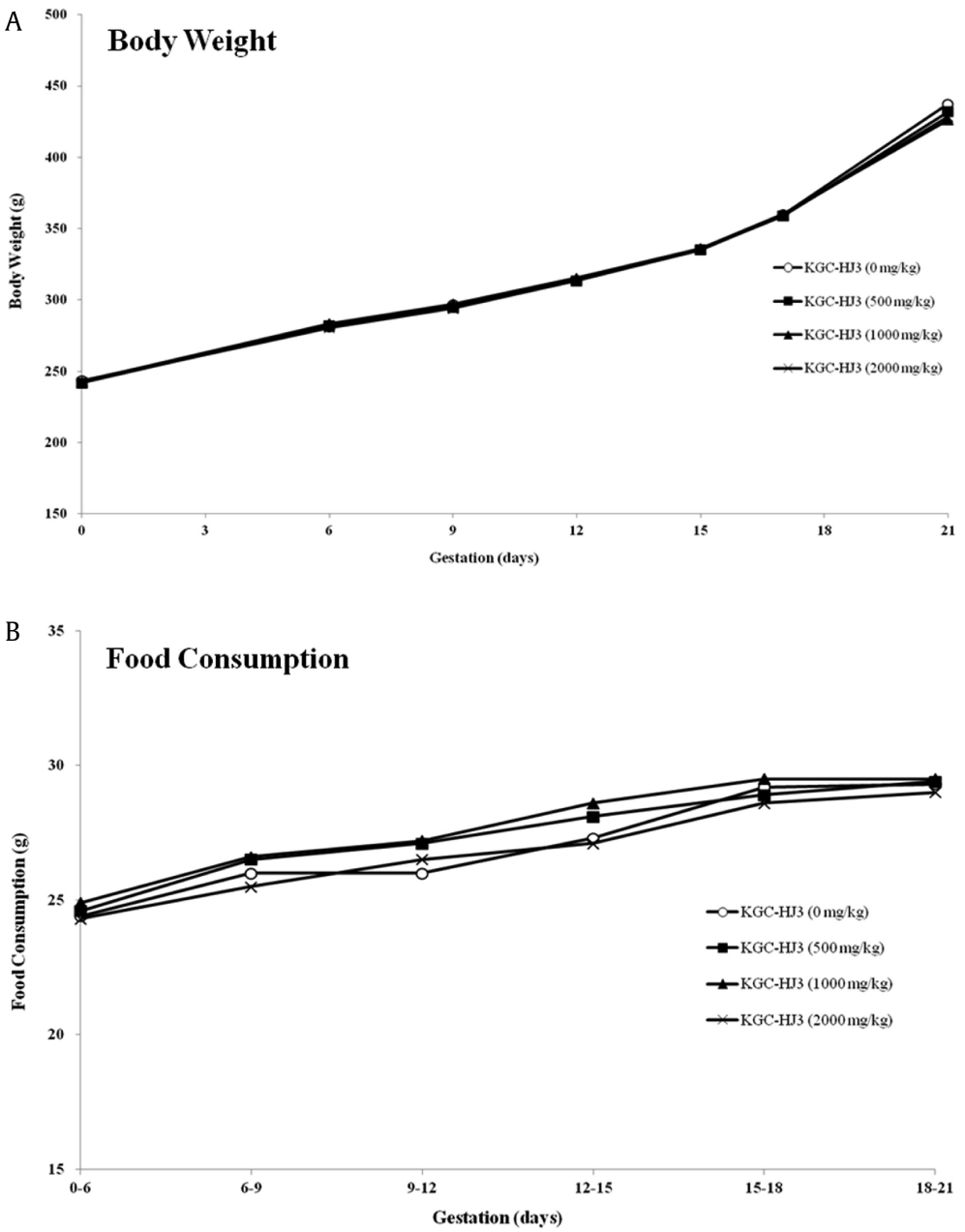


Fig. 3. Mean body weight changes and food consumption of rats in embryo-fetal development study. (A) Body weight change. (B) Food consumption. Four groups were treated with KGC-HJ3 at dose levels 0 (○), 500 (■), 1000(▲), and 2000 (×) mg/kg. The rats treated with KGC-HJ3 showed no significant difference compared with the control.

Table 4
Organ weights and gravid uterine-related results of females treated with KGC-HJ3 in embryo-fetal development study

Parameter	KGC-HJ3 (mg/kg)			
	0	500	1000	2000
No. of pregnant females	18	21	20	20
Terminal body weight (TBW)	429.6 ± 23.5[1)]	424.4 ± 20.5	419.0 ± 37.6	421.8 ± 31.3
Liver (g)	16.22 ± 1.43	15.91 ± 1.50	16.14 ± 1.60	15.88 ± 1.83
Per terminal body weight (%)	3.78 ± 0.26	3.75 ± 0.30	3.86 ± 0.29	3.77 ± 0.35
Spleen (g)	0.67 ± 0.12	0.64 ± 0.09	0.64 ± 0.10	0.66 ± 0.12
Per terminal body weight (%)	0.16 ± 0.03	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.02
Heart (g)	1.05 ± 0.07	1.05 ± 0.09	1.06 ± 0.11	1.03 ± 0.11
Per terminal body weight (%)	0.24 ± 0.01	0.25 ± 0.02	0.26 ± 0.03	0.24 ± 0.02
Lung (g)	1.29 ± 0.10	1.28 ± 0.09	1.31 ± 0.15	1.27 ± 0.12
Per terminal body weight (%)	0.30 ± 0.02	0.30 ± 0.02	0.31 ± 0.03	0.30 ± 0.02
Kidneys (g)	2.19 ± 0.23	2.14 ± 0.18	2.25 ± 0.20	2.23 ± 0.25
Per terminal body weight (%)	0.51 ± 0.05	0.50 ± 0.04	0.54 ± 0.06	0.53 ± 0.04
Adrenal gland (g)	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Per terminal body weight (%)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Brain (g)	1.91 ± 0.10	1.87 ± 0.09	1.89 ± 0.09	1.89 ± 0.08
Per terminal body weight (%)	0.45 ± 0.03	0.44 ± 0.03	0.46 ± 0.04	0.45 ± 0.04
Pituitary gland (g)	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Per terminal body weight (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Right ovary (g)	0.07 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.02
Per terminal body weight (%)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Left ovary (g)	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
Per terminal body weight (%)	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Gravid uterine weight (g)	108.7 ± 19.2	103.7 ± 12.0	92.8 ± 27.4	99.9 ± 19.0
Corrected terminal weight (g)[2)]	328.9 ± 14.4	328.4 ± 14.4	333.7 ± 22.2	328.6 ± 20.0
Net body weight change (g)[3)]	48.2 ± 12.1	46.4 ± 12.7	50.3 ± 12.5	48.2 ± 11.2

[1)] Values are presented as mean ± standard deviation.
[2)] Body weight on GD 21 − Gravid uterine weight.
[3)] Corrected terminal body weight − Body weight on GD 6.

was not considered treatment related, given the lack of dose dependency and no related fertility indices change and histopathological abnormalities.

3.2. Embryo-fetal development study

There was no treatment-related mortality and clinical sign abnormalities in embryo-fetal development study. In body weight and food consumption observations (Fig. 3), there were no treatment-related changes in any of the treatment groups. At terminal observation, there were no treatment-related changes in macroscopic findings (data not shown), organ weights, gravid uterine weights, corrected terminal body weight, and net body weight change (Table 4).

In embryo-fetal development, there were no treatment-related changes, including Caesarean section and fetal external, visceral, and skeletal examinations (Tables 5 and 6). In Caesarean section, a statistically significant decrease in the number of corpora lutea at 1000 mg/kg was observed, but it was considered incidental findings because treatment was initiated after the corpora lutea

Table 5
Caesarean section data of females treated with KGC-HJ3 in embryo-fetal development study

Parameter	KGC-HJ3 (mg/kg)			
	0	500	1000	2000
No. of pregnant animals	18	21	20	20
No. of corpora lutea	16.1 ± 2.3[1)]	15.5 ± 2.0	14.6 ± 1.2*	15.1 ± 1.6
No. of implantations	15.3 ± 2.8	14.6 ± 1.7	12.9 ± 3.6	14.2 ± 2.6
No. of fetal deaths (resorptions + dead fetuses)	0.6 ± 0.9	0.4 ± 0.6	0.8 ± 1.6	0.6 ± 0.8
No. of early resorption	0.4 ± 0.6	0.4 ± 0.6	0.7 ± 1.6	0.5 ± 0.8
No. of late resorption	0.1 ± 0.4	0.0 ± 0.2	0.1 ± 0.2	0.1 ± 0.2
No. of dead fetuses	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Postimplantation loss (%)[2)]	4.1 ± 5.8	2.9 ± 4.0	5.6 ± 11.7	4.1 ± 5.7
Litter size (No. of live fetuses)	14.7 ± 2.9	14.1 ± 1.7	12.2 ± 3.8	13.6 ± 2.8
Sex ratio (mean % of male per litter)	48.4 ± 11.6	51.8 ± 15.2	55.0 ± 9.9	50.9 ± 16.2
Mean fetal weight (covariate-adjusted mean)				
Total	5.67	5.56	5.72	5.58
Male	5.83	5.70	5.84	5.72
Female	5.54	5.38	5.56	5.44
Mean placental weight (covariate-adjusted mean)				
Total	0.50	0.49	0.50	0.48
Male	0.51	0.50	0.51	0.49
Female	0.49	0.48	0.50	0.47

* Significant difference at $p < 0.05$ level compared with the control group.
[1)] Values are presented as mean ± standard deviation.
[2)] [(No. of implantation − No. of live fetuses)/No. of implantation] × 100.

Table 6
Selected fetal morphological examinations results treated with KGC-HJ3 in embryo-fetal development study.

Parameter	KGC-HJ3 (mg/kg)			
	0	500	1000	2000
Fetal external examination				
No. of litters examined	18	21	20	20
No. of fetuses examined	264	297	243	271
No. of litters with external malformations (%)	0 (0)	0 (0)	0 (0)	1 (5.0)
No. of fetuses with external malformations (%)	0 (0)	0 (0)	0 (0)	1 (0.3)
Bent tail	0	0	0	1
Fetal visceral examination				
No. of litters examined	18	21	20	20
No. of fetuses examined	127	143	116	131
No. of litters malformations (%)	1 (5.5)	0(0.0)	0(0.0)	0(0.0)
No. of fetuses with malformations (%)	1 (0.8)	0(0.0)	0(0.0)	0(0.0)
Aortic arch, supernumerary branch	1	0	0	0
No. of litters variations (%)[1)]	17(94.4)	18(85.7)	16(80.0)	19(95.0)
No. of fetuses with variations (%)[2)]	70(55.1)	48(33.6)**	42(36.2)**	45(34.4)**
Brain, dilated lateral ventricle	0	1	0	0
Thymus, Misshapen	38	18**	10**	10**
Ureters, convoluted	37	23*	23	21*
Ureters, dialted	15	15	16	15
Fetal skeletal examination				
No. of litters examined	18	21	20	20
No. of fetuses examined	137	154	127	140
No. of litters malformations (%)	0(0.0)	0(0.0)	0(0.0)	1(5.0)
No. of fetuses with malformations (%)	0(0.0)	0(0.0)	0(0.0)	1(0.7)
Ribs, Absent	0	0	0	1
Ribs, Short	0	0	0	1
No. of litters variations (%)[1)]	8(44.4)	10(47.6)	12(60.0)	11(55.0)
No. of fetuses with variations (%)[2)]	10(7.3)	18(11.7)	18(14.2)	19(13.6)
Sternebrae, bipartite ossification	0	1	0	2
Sternebrae, misaligned	0	1	0	2
Ribs, full supernumerary	0	1	1	0
Ribs, short supernumerary	8	7	11	5
Interparietal, Incomplete ossification	0	2	0	1
Parietal, Incomplete ossification	1	6	1	0
Supraoccipital, Incomplete ossification	0	1	1	1
Zygomatic, Incomplete ossification	0	0	0	1
Thoracic centrum, bipartite ossification	1	2	0	1
Thoracic centrum, dumbbell ossification	1	1	5	8*
Pubis, incomplete ossification	0	0	0	1
No. of ossification centers[3)]				
Sternebra	6.0±0.0	6.0±0.1	6.0±0.0	6.0±0.0
Metacarpals in both forelimbs	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.1
1st phalanges in both forelimbs	5.8±1.5	5.2±1.7	5.2±1.3	4.5±1.8
Metatarsals in both hindlimbs	9.7±0.4	9.7±0.5	9.7±0.5	9.5±0.5
1st phalanges in both hindlimbs	1.7±1.5	1.7±1.9	1.8±1.9	1.0±1.1
Cervical vertebra	5.5±1.1	4.9±1.2	5.5±1.3	5.0±1.4
Sacral and caudal vertebra	10.2±0.7	10.2±0.6	10.4±0.7	9.9±0.7

* Significant difference at $p < 0.05$ level compared with the control group.
** Significant difference at $p < 0.01$ level compared with the control group.
1) Includes litters with one or more affected fetuses.
2) A single fetus may be presented more than once in listing individual defects.
3) Values are presented as mean ± standard deviation.

formation in this type of embryo-fetal development study. In fetal morphological examinations, a statistically significant decrease in misshapen thymus and convoluted ureter as visceral variation at all treated groups was determined but was not considered treatment related because the incidence of these variations at control group was incidentally increased when compared with our institutional historical control data (2007–2014, mean incidence of misshapen thymus: 10.4%, mean incidence of convoluted ureter: 4.2%). A bent tail as external malformation in one fetus and complex skeletal malformation including absent rib and short rib in one fetus at 2000 mg/kg were observed. However, they were not considered treatment related because the incidence was very low, and there was no other related change in other parameters in this group. A statistically significant increase in the dumbbell ossification of thoracic centrums at 2000 mg/kg was not considered treatment related because there was no change in other skeletal ossification retardation findings, such as the decreased number of ossification centers, fetal weight, and so forth.

4. Discussion and conclusion

A fertility and early embryonic development study was conducted in 176 SD rats (22 males and 22 females per group) to determine the potential reproductive toxicity of KGC-HJ3 at dose levels with 0 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg. All animals grew normally and survived to scheduled sacrifice, and there was no treatment-related change observed in general clinical signs, body weight, weight gain, food consumption, macroscopic observation, organ weights, and histopathological observation of reproductive organs. In addition, no potential reproductive toxicity findings were observed in estrus cycle, sperm analysis, precoital time, fertility-related indices, and Caesarean section results on GD

15. In addition, an embryo-fetal development study was conducted in 88 mating-proven SD rats (22 females per group) to determine the potential maternal and embryo-fetal development toxicity including teratogenic potential of KGC-HJ3 at dose levels with 0 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg. There was no maternal toxicity observed in general clinical sign, body weight, and weight gain, food consumption, macroscopic observation, and organ weights. In addition, no embryo-fetal development toxicity and teratogenic potentials were observed in Caesarean section results on GD 21 and fetal morphological examinations.

Angelica gigas is one of the three types *Angelica* roots which can be found in East Asian countries as the important component of the traditional folk medicine [24]. In fact, previous studies demonstrated its pharmacological effects in various diseases condition [25]; however, there is lack of extensive toxicity studies for its safety evaluation. A 13-week repeated dose oral gavage toxicity study demonstrated that the no observed adverse effect level (NOAEL) of *Angelica gigas* was considered to be at least 2000 mg/kg/day. In addition, a battery of genotoxicity studies including Ames test, *in vitro* chromosome aberration assay, and *in vivo* micronucleus assay suggested that *Angelica gigas* was not genotoxic [26]. However, the safety evaluation for its potential adverse effect on developmental and reproductive function was not investigated so far.

Deer antlers also have been used in traditional folk medicine in East Asian countries because of its pharmacological effects for osteoporosis, cardiovascular, gynecological, and immunological (e.g., tuberculosis, arthritis etc.) disease [27,28]. Although there were no extensive toxicological data for *deer antlers* considering its extensive usage, a previous 13-week repeated dose oral gavage toxicity study demonstrated that the NOAEL was considered to be at least 1000 mg/kg/day [29]. However, there were also no toxicological data for its potential adverse effect on developmental and reproductive function.

Korean Red Ginseng is produced by heating and drying the root of *Panax ginseng* Meyer (Araliaceae), and it is widely used in traditional folk medicine as well as nutritional supplement, cosmetics, and medicinal components [30]. Although several toxicological studies with ginseng demonstrated that it is a safety component for human health, there were also controversial results for its safety. A 4-week repeated dose oral gavage toxicity study with rat demonstrated that the NOAEL of Korean Red Ginseng was considered to be at least 2000 mg/kg/day [31]. In addition, an embryo-fetal development study with mice demonstrated that the NOAEL of Korean Red Ginseng was also considered to be at least 2000 mg/kg/day [32]. However, embryotoxic effect of the ginseng component, ginsenosides Re, was reported in rat whole embryo culture model [33]. In addition, another ginsenoside Rb1 induced morphological change in the same experimental condition [34]. For this reason, it is recommended to consume ginseng with caution during pregnancy, especially during the first trimester and during lactation [35].

A mixture exposure of combined materials is a real-life human exposure scenario for human risk assessment. However, human risk assessment using toxicological results with experimental animals predominantly has been carried out on the single substances exposure to human. Combined action of concurrently exposed chemicals is generally defined as additive, synergistic, or antagonistic [36]. Additive means no interaction among exposed chemicals. Synergistic means enhancement of toxicity, and antagonistic means inhibition of toxicity. Recently, many researchers are interested in the field of mixture toxicology, and several previous studies for mixture toxicology and their risk assessment have been conducted [37–39].

In this study, individual components of KGC-HJ3, Korean Red Ginseng, *Angelica gigas*, and *deer antlers*, were reported as relatively nontoxic in the previous repeated-toxicity studies, but their mixture exposure effect was not sufficiently evaluated. In particular, there was no published information of the safety assessment in reproductive and developmental aspects for A*ngelica gigas* and *deer antlers*. In addition, some ginsenosides, such as Rb1 and Re, were reported embryotoxic in rat whole embryo culture model. However, based on the results of this study, combined exposure of KGC-HJ3 was considered nontoxic for developmental and reproductive function in rats. There were no toxicity findings even at very high dose (2000 mg/kg/day) in fertility/early embryonic development study and embryo-fetal development study with rats.

In conclusion, the safety assessment of KGC-HJ3 in developmental and reproductive function was conducted, and combined exposure of Korean Red Ginseng, *Angelica gigas*, and *deer antlers* was considered nontoxic. The NOAELs of KGC-HJ3 for fertility, early embryonic development, maternal function, and embryo-fetal development is considered to be at least 2000 mg/kg/day.

Conflicts of interest

Byungcheol Han and Yong-Soon Kim are current or former employees of the Korea Ginseng Corporation that are responsible for the development and marketing of KGC-HJ3. The others declare that there are no conflicts of interest.

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References

[1] Jeon BH, Kim CS, Kim HS, Park JB, Nam KY, Chang SJ. Effect of Korean red ginseng on blood pressure and nitric oxide production. Acta Pharmacol Sinica 2000;21:1095–100.
[2] Kaneko H, Nakanishi K. Proof of the mysterious efficacy of ginseng: basic and clinical trials: clinical effects of medical ginseng, Korean red ginseng: specifically, its anti-stress action for prevention of disease. J Pharmacol Sci 2004;95:158–62.
[3] Park JG, Son YJ, Aravinthan A, Kim JH, Cho JY. Korean red ginseng water extract arrests growth of xenografted lymphoma cells. J Ginseng Res 2016;40:431–6.
[4] Kim HJ, Chae IG, Lee SG, Jeong HJ, Lee EJ, Lee IS. Effects of fermented red ginseng extracts on hyperglycemia in streptozotocin-induced diabetic rats. J Ginseng Res 2010;34(2):104–12.
[5] Lee YK, Chin YW, Choi YH. Effects of Korean red ginseng extract on acute renal failure induced by gentamicin and pharmacokinetic changes by metfomin in rats. Food Chem Toxicol 2013;59:153–9.
[6] Qureshi AA, Abuirmeileh N, Din ZZ, Ahmad Y, Burger WC, Elson CE. Suppression of cholesterogenesis and reduction of LDL cholesterol by dietary ginseng and its fractions in chicken liver. Atherosclerosis 1983;48(1):81–94.
[7] Joo SS, Park D, Shin S, Jeon JH, Kim TK, Choi YJ, Lee SH, Kim JS, Park SK, Hwang BY, et al. Anti-allergic effects and mechanisms of action of the ethanolic extract of *Angelica gigas* in dinitrofluorobenzene-induced inflammation models. Environ Toxicol Pharmacol 2010;30:127–33.
[8] Lee YY, Lee S, Jin JL, Yun-Choi HS. Platelet anti-aggregatory effects of coumarins from the roots of *Angelica genuflexa* and *A. gigas*. Arch Pharm Res 2003;26:723–6.
[9] Gilbey A, Perezgonzalez JD. Health benefits of deer and elk velvet antler supplements: a systematic review of randomised controlled studies. NZ Med J 2012;125(1367):80–6.
[10] Kawtikwar PS, Bhagwat DA, Sakarkar DM. Deer antlers-traditional use and future perspectives. Indian J Tradit Knowl 2010;9:245–51.
[11] Liu P, Xu YJ, Yin HJ, Zhang ZF, Wang JB, Chen KJ, Li Y. Effects of ginsenoside Rb1 on mouse embryonic development in vitro. J Hygiene Res 2005;34:175–7.

[12] Chan LY, Chiu PY, Lau TK. Embryotoxicity study of ginsenoside Rc and Re in vitro rat whole embryo culture. Reprod Toxicol 2004;19:131–4.
[13] Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the care and use of laboratory animals. 8th ed. Washington DC: National Academies Press; 2011.
[14] Ministry of Food and Drug Safety (MFDS) Notification No. 2013-40. Good laboratory Practice regulations for Nonclinical laboratory studies. 2013.
[15] Organization for Economic Co-operation and Development (OECD). Principles of Good laboratory Practice. 1997.
[16] Ministry of Food and Drug Safety (MFDS) Notification No. 2013-121. Test guidelines for safety evaluation of drugs annex 3 reproductive and development toxicology. 2013.
[17] International Council for Harmonisation (ICH) Harmonized Tripartite Guidelines S5 (R2). Detection of toxicity to reproduction for medicinal products & toxicity to male fertility. 2005.
[18] Salewski E. Faerbermethode zum stellen am uterus der ratte. Naunyn-Schmeidebergs Arch Pharmakol. Exp Toxicol 1964;247:367.
[19] Wilson JG. Methods for administering agents and detecting malformations in experimental animals. In: Wilson JG, Warkany J, editors. Teratology, principles and techniques. Chicago and London: University of Chicago Press; 1965.
[20] Nishimura K. A microdissection method for detecting thoracic visceral malformations in mouse and rat fetuses. Cong Anom 1974;14:23–40.
[21] Staples RE. Detecting of visceral alterations in mammalian fetuses. Teratology 1974;9:37–8.
[22] Dawson AB. A note on the staing of the skeleton of cleared specimens with Alizarin Red. S. Stain Technol 1926;1:123–4.
[23] Makris SL, Solomon HM, Clark R, Shiota K, Barbellion S, Buschmann J, Ema M, Fujiwara M, Grote K, Hazelden KP, et al. Terminology of developmental abnormalities in common laboratory mammals (Version 2). Birth Defects Res. B Dev Reprod Toxicol 2009;86:227–327.
[24] Lv N, Koo JH, Yoon HY, Yu J, Kim KA, Choi IW, Kwon KB, Kwon KS, Kim HU, Park JW, et al. Effect of *Angelica gigas* extract on melanogenesis in B16 melanoma cells. Int J Mol Med 2007;20:763–7.
[25] Jang JY, Kim J, Cai J, Kim Y, Shin K, Kim TS, Lee SP, Park SK, Choi EK, Kim YB. An ethanolic extract of *Angelica gigas* improves atherosclerosis by inhibiting vascular smooth muscle cell proliferation. Lab Anim Res 2014;30:84–9.
[26] Yun JW, Che JH, Kwon E, Kim YS, Kim SH, You JR, Kim WH, Kim HH, Kang BC. Safety evaluation of *Angelica gigas*: genotoxicity and 13-weeks oral subchronic toxicity in rats. Regul Toxicol Pharmacol 2015;72:473–80.
[27] Huang TK. Handbook of composition and pharmacological action of commonly-used traditional Chinese medicine. 2nd ed. Beijing: China Medical and Pharma-ceutical Science Publishing House Press; 1997.
[28] Chen JC, Hsiang CY, Lin YC, Ho TY. Deer antler extract improves fatigue effect through altering the expression of genes related to muscle strength in skeletal muscle of mice. Evid Based Compl Alt 2014:10. Article ID 540580.
[29] Zhang H, Wanwimolruk S, Coville PF, Schofield JC, Williams W, Haines SR, Suttie JM. Toxicological evaluation of New Zealand deer velvet powder. Part I: acute and subchronic oral toxicity studies in rats. Food Chem Toxicol 2000;38:985–90.
[30] Kaneko H, Nakanishi K. Proof of the mysterious efficacy of ginseng: basic and clinical trials: clinical effects of medical ginseng, Korean red ginseng: specifically, its anti-stress action for prevention of disease. J Pharmacol Sci 2004;95:158–62.
[31] Park SJ, Lim KH, Noh JH, Jeong EJ, Kim YS, Han BC, Lee SH, Moon KS. Subacute oral toxicity study of Korean red ginseng extract in Sprgue-Dawely rats. Toxicol Res 2013;29:285–92.
[32] Shin S, Jang JY, Park D, Yon JM, Beak IJ, Hwang BY, Nam SY, Yun YW, Kim KY, Joo SS, et al. Korean red ginseng extract does not cause embryo- fetal death or abnormalities in mice. Birth Defects Res B 2010;89:78–85.
[33] Chan LY, Chiu PY, Lau TK. An in-vitro study of ginsenoside Rb1-induced teratogenicity using a whole rat embryo culture model. Human Reprod 2003;18:2166–8.
[34] Liu P, Xu Y, Yin H, Wang J, Chen K, Li Y. Developmental toxicity research of ginsenoside Rb1 using a whole mouse embryo culture model. Birth Defects Res B 2005;74:207–9.
[35] Seely D, Dugoua JJ, Perri D, Mills D, Koren G. Safety and efficacy of Panax ginseng during pregnancy and lactation. Can J Clin Pharmacol 2008;15:e87–94.
[36] Könemann WH, Pieters MN. ConfusNion of concepts in mixture toxicology. Food Chem Toxicol 1996;34:1025–31.
[37] Kortenkamp A. Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. Curr Opin Pharmacol 2014;19:105–11.
[38] Thomas B, Michael F. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environ Sci Technol 2012;46:2564–73.
[39] Boaretoa AC, Müllera JC, Araujoa SL, Lourenc AC, Lourenc EL, Gomes C, Minatovicz B, Lombardi N, Paumgartten FR, Dalsenter PR. Study on the developmental toxicity of combined artesunate and mefloquine antimalarial drugs on rats. Reprod Toxicol 2012;34:658–64.