Original Article

Korean Red Ginseng Extract Does Not Cause Embryo-Fetal Death or Abnormalities in Mice

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BACKGROUND: Ginseng has been used for a long time and is well tolerated in humans. However, recent studies have shown that ginsenosides Rb1, Rg1, and Re exert embryotoxicity in in vitro culture systems. We investigated the effects of Korean red ginseng extract (KRGE) on embryonic implantation and fetal development in mice. **METHODS:** Mice were orally administered KRGE (20, 200, or 2,000 mg/kg/day) from 2 weeks before mating to gestational day (GD) 18, and implantation rate, fetal mortality, body weights, as well as external, visceral, and skeletal abnormalities were determined by Caesarean section on GD18. Ginsenosides in KRGE and in the blood of dams were identified and quantified by HPLC analysis. **RESULTS:** KRGE did not affect embryonic implantation and mortality as well as fetal body weights up to 2,000 mg/kg/day (≈200 times clinical doses), the upper-limit dose recommended by the Korea Food and Drug Administration (KFDA). Although the prevalence of supernumerary ribs increased at the medium dose (200 mg/kg/day), no dose-dependent increases in external, visceral, and skeletal abnormalities were observed. Major ginsenosides such as Rb1, Rg1, and Re were not detected in the blood of dams based on their chromatographic profiles. **CONCLUSIONS:** Considerable developmental toxicities of KRGE, even at the upper-limit dose, were not observed in mice. These results might be due to the negligible blood concentrations of ginsenosides in their original forms following oral administration, suggesting that in vitro experiments to assess the effects of ginsenosides on embryotoxicity may not reliably explain the risks of ginsenosides to in vivo embryo-fetal development. *Birth Defects Res (Part B)* 89:78–85, 2010. © 2010 Wiley-Liss, Inc.

Key words: ***Panax ginseng; ginsenosides; protopanaxadiols; protopanaxatriols; developmental toxicity***

INTRODUCTION

Ginseng is a representative herbal remedy used in Oriental medicine and has been used as a dietary supplement worldwide. Korean red ginseng is produced by heating and drying the root of *Panax ginseng* C.A. Meyer (Araliaceae), and its effects are now widely recognized. It has been shown to exert pharmacological functions such as anti-diabetic, anti-hypertensive, anti-stress, aphrodisiac, antioxidant, anti-tumor, and memory-enhancing activities, among others (Jeon et al., 2000; Kaneko and Nakanishi, 2004; Keum et al., 2000; Nocerino et al., 2000; Scholey and Kennedy, 2002; Seely et al., 2008; Sotaniemi et al., 1995).

The chemical ingredients of ginseng were well defined and ginseng saponins, called ginsenosides, are considered to be the active component of ginseng. Over 40 different ginsenosides were identified and classified into several types by the presence of special chemical structures (protopanxadiol, propanaxatriol, and oleanolic acid) (Attele et al., 1999; Kowalski, 2007; Tawab et al., 2003) and account for most of ginseng's efficacy. An earlier report (Liberti and Der Marderosian, 1978) suggested that the ingredients of ginseng vary depending on the ginseng species, cultivation period, and methods of production. In addition, the pharmacological effect of ginseng also depends on the ginseng type (Sievenpiper et al., 2003).

Ginseng has been used for thousands of years and is generally considered to be safe, but it has been reported that ginseng has some adverse effects (Ernst, 2002). Especially, recent studies have shown that some ingredients of ginsenosides such as Rb1, Rg1, and Re induced abnormalities in in vitro embryo culture (Chan et al., 2003, 2004; Liu et al., 2005, 2006). These studies suggest

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that ginseng can be teratogenic. Notably, there is evidence demonstrating the presence of major ginsenosides including Rb1 in the plasma and urine following oral administration (Ji et al., 2004; Tawab et al., 2003), suggesting that these ginsenosides may produce developmental side effects. Although it was reported that the use of ginseng did not exhibit adverse effects on pregnancy outcomes (Holst et al., 2008; Seely et al., 2008), it was not ruled out that, at high concentrations, ginsenosides Rb1, Rg1, and Re could be delivered into the uterus and affect the embryo or fetal development.

However, since the previous embryotoxicity results were based on in vitro experiments, the safety issue of ginseng during embryogenesis and fetal development needs to be verified in vivo. In the present study, we evaluated the developmental toxicity of KRGE up to an extremely-high dose; 2,000 mg/kg/day ($\approx$200 times clinical doses), the upper-limit dose recommended by the KFDA.

MATERIALS AND METHODS

KRGE Preparation and Ginsenosides Quantification

To assess the toxicity of KRGE in fetal development, 9 kg of KRGE was pulverized and boiled with distilled water at 100°C for 3 h. The process was repeated 3 times and the collected solution was filtered through a Whatman filter paper. The solution was concentrated in a vacuum evaporator and then pulverized after lyophilization. To make standard solutions, 10, 100, 500, 1,000, and 2,000 μg of major ginsenosides Rb1 and Rg1 were dissolved in methanol and filtered through a 0.45-μm filter. Twenty micrograms of each standard solution was analyzed by HPLC and a calibration curve was determined to calculate the amount of Rb1 and Rg1 in a sample. Chromatographic separation was achieved using YMC-ODS H80 (150 × 4.6 mm internal diameter) and photodiode detection was employed at a wavelength of 203 nm. Acetonitrile was used as the mobile phase and water was used with a gradient system (25:75 to 45:55) for 30 min. The flow rate was 1 ml/min.

Animals and Treatment

Seven-week-old female and 5-week-old male ICR mice were procured from Samtako (Osan, Korea). Animals were maintained at a constant temperature (23 ± 2°C), relative humidity of 50–60%, and 12-h light/dark cycle in the Laboratory Animal Research Center at Chungbuk National University, Korea. Standard rodent chow and purified water were available ad libitum. All experimental procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center at Chungbuk National University. After acclimation for 1 week to the laboratory environment, female mice were orally administered KRGE, dissolved in purified water, at dose levels of 20, 200, or 2,000 mg/kg/day ($n = 25$ per each dose level) by gavage in a volume of 5 ml/kg from 2 weeks before mating to gestational day 18 (GD18). The control group was treated with vehicle alone. Female and male mice (1:1) were coupled every evening (18:00) for 5 nights, and GD0 was determined by observation of a vaginal plug in the morning of the next day (07:00–08:00).

Measurement of Blood Concentration of Ginsenosides

Blood concentrations of ginsenosides were determined using a previously described quantification method (Li et al., 2004; Xu et al., 2003). Briefly, serum samples were collected on GD18 and applied to SPE cartridge for HPLC analysis. A flavonoid genistein was used as the internal standard. Genistein and a ginsenosides mixture [Rb1, Rg1, Re, and ginseng total extract (Gte)] dissolved in acetonitrile-water mixture (600 μl) were applied to glass tubes and solvents were removed by N_2 gas. Serum (2 ml) was added into each tube and loaded in Extract-clean TM C18 column eluted with water (5 ml) and methanol (5 ml). Cartridges were washed with water (5 ml) and 10% methanol (2 ml) and dried with N_2 gas after elution with 70% methanol, followed by dissolving with 300 μl of 50% acetonitrile. Twenty microliters was used for HPLC analysis.

Toxicity Assessment

During treatment, body weight and feed consumption of pregnant females ($n = 18$–20 per group) were measured every other day, and their clinical signs were recorded every day. Dams were euthanized under deep anesthesia with ethyl ether on GD18, and fetal abnormalities were determined by Caesarean section. The placentas were removed and weighed, and the live, dead, and resorbed fetuses were counted. Live fetuses were weighed and examined for possible external abnormalities. After external examination, the fetuses were divided into 2 groups for visceral and skeletal examination. One group of fetuses was fixed in Bouin's solution for 1 week, and subsequently sliced to detect visceral abnormalities (Nishimura, 1974; Wilson, 1965). To determine skeletal malformation, the other group of fetuses was fixed in 95% ethanol, stained with Alizarin red S and Alcian blue, and examined under a stereomicroscope (Dawson, 1926; Manson and Kang, 1989). Delayed ossification was defined as lack of Alizarin red–stained parietal bone (cranium) and/or one or more sternebrae (sternum) (Park et al., 2009).

Statistical Analysis

Statistical analyses were performed using SAS software (Cary, NC). Continuous variables such as maternal body weight, fetal body weight, and placental weight were compared by one-way analysis of variance (ANOVA), followed by Scheffe's multiple comparison test when the results were significant. The number of implantations, live fetuses, and dead fetuses was compared using Kruskal-Wallis nonparametric ANOVA, followed by a Mann-Whitney U-test. Incidence data, such as external and visceral abnormalities, were compared using Fisher's exact probability test. Statistical differences were considered significant when $P < 0.05$.

RESULTS

Embryo-Fetal Toxicity

KRGE (20–2,000 mg/kg/day) orally treated from 2 weeks before mating neither affected maternal body weights (Fig. 1) and feed consumption (data not shown), nor induced clinical signs or abortion. Also, KRGE did

not influence embryonic implantation, showing 11.24–13.94 of implantation sites in vehicle or KRGE-treated mice (Table 1). In addition, continuous treatment of KRGE to the end stage of gestation did not increase resorption or death of embryos and fetuses, although the mortality rate tended to decrease in the high dose (2,000 mg/kg/day) group. KRGE also did not influence the fetal and placental weights.

Although the low (20 mg/kg/day) and medium (200 mg/kg/day) doses of KRGE slightly increased the incidence of external abnormalities, the rate was not statistically significant (Table 2). The abnormalities included edema and hematoma at both doses, as well as 3 cases of exencephaly in a litter exposed to 200 mg/kg/day. However, a higher dose (2,000 mg/kg/day) of KRGE did not induce such abnormalities.

Incidences of the renal pelvic dilatation slightly increased in fetuses exposed to the medium dose (200 mg/kg/day) of KRGE (Table 3). However, there was no evidence of dose-dependent increases in the incidences of visceral abnormalities: cleft palate, thymic abnormalities, and anophthalmia.

Administration of KRGE (200 mg/kg/day) appeared to significantly increase skeletal abnormalities, mainly inducing short supernumerary (14th) ribs (Table 4). In addition, 200 mg/kg/day of KRGE tended to increase the incomplete ossification of the sternum. However, both lower (20 mg/kg/day) and higher doses (2,000 mg/kg/day) of KRGE did not increase such costal abnormalities and sternal incomplete ossification.

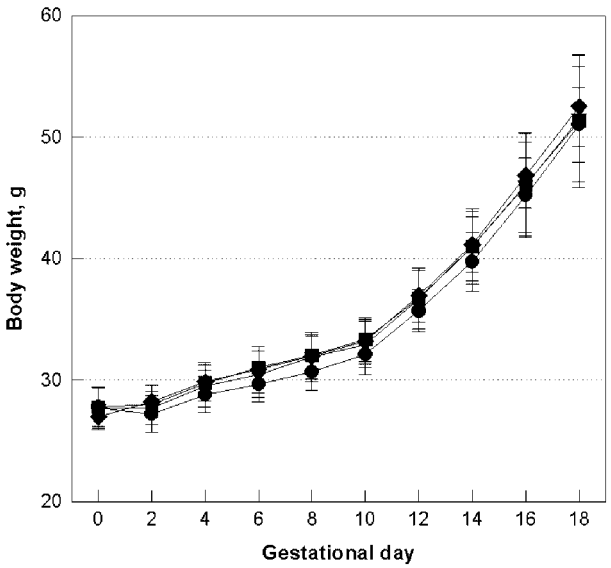

Fig. 1. Change in body weights of pregnant female mice orally administered with KRGE 2 weeks before mating. •, vehicle (water, 5 ml/kg/day) alone; ▼, KRGE 20 mg/kg/day; ■, KRGE 200 mg/kg/day; ♦ KRGE 2,000 mg/kg/day.

Quantification of Ginsenosides

Rb1 and Rg1 standard solutions were prepared and the calibration curves of major ginsenosides Rb1 and Rg1 were determined by HPLC anaylsis. The retention times for Rg1 and Rb1 were 7.5 and 20.1 min, respectively (Fig. 2A and B). Figure 1C shows the chromatographic profile of KRGE. Using the calibration curves, concentrations of Rb1 and Rg1 in KRGE were determined to be 0.61 ± 0.12 and $0.90 \pm 0.17\%$, respectively.

Blood from dams on GD18 that were orally administered KRGE 2 weeks prior to mating was collected, and the serum concentrations of Rb1, Rg1, and Re were determined by HPLC analysis. In comparison with the detection of each ginsenoside after spiking into normal serum (Fig. 3A), no trace of these ginsenosides was observed in the serum samples 30 min (Fig. 3B) and 2 h (Fig. 3C) following treatment with the highest dose (2,000 mg/kg/day) of KRGE.

DISCUSSION

It has been well known that ginseng is generally well tolerated and its adverse effects are very rare in most people if used at recommended doses (Chang et al., 2003; Seely et al., 2008). However, previous studies showed that inappropriate use of ginseng induced some adverse effects including mild hypertension (Siegel, 1979; Seely et al., 2008), and interaction with other drugs, such as anticoagulants, has been reported (Fugh-Berman, 2000; Janetzky and Morreale, 1997; Seely et al., 2008). Thus, the safety of ginseng remains an open question. Especially, it is notable that the exposure of mouse and rat embryos in vitro to Rb1, Rg1, and Re caused growth retardation and malformations including a decreased median total morphological score (Chan et al., 2003, 2004; Liu et al., 2005, 2006). Therefore, reliable studies are

Table 1
Implantation, Mortality and Body Weight of Fetuses, Examined on Gestational Day 18, From Dams Orally Administered With Korean Red Ginseng Extract (KRGE)

Treatment (mg/kg/day)	Vehicle	KRGE (20)	KRGE (200)	KRGE (2,000)
No. of dams used	19	19	20	18
Implantation sites	12.53 ± 3.81	11.24 ± 3.24	12.85 ± 2.66	13.94 ± 6.70
Live fetuses[a]	11.83 ± 2.62	9.84 ± 4.07	11.80 ± 3.61	13.06 ± 2.44
Resorption and death[a]	1.34 ± 1.31	1.50 ± 2.48	1.33 ± 2.42	0.92 ± 0.87
Rate (%)[a]	10.67 ± 10.45	13.35 ± 23.94	10.37 ± 20.30	6.57 ± 6.12
Fetal body weight (g)				
Male	1.42 ± 0.13	1.43 ± 0.13	1.42 ± 0.13	1.44 ± 0.30
Female	1.36 ± 0.12	1.39 ± 0.11	1.34 ± 0.13	1.35 ± 0.10
Placental weight (g)	0.10 ± 0.01	0.12 ± 0.04	0.10 ± 0.01	0.10 ± 0.01

[a]Indicates mean number or percentage of fetuses affected per litter.

Table 2
External Abnormalities of Fetuses From Dams Orally Administered With Korean Red Ginseng Extract (KRGE)

Treatment (mg/kg/day)	Vehicle	KRGE (20)	KRGE (200)	KRGE (2,000)
No. of dams used	19	19	20	18
No. of fetuses examined/litter	11.83±2.62	9.84±4.07	11.80±3.61	13.06±2.44
Total No. of fetuses examined	213	187	236	235
Abnormalities observed[a]	0.26±0.45	0.79±1.32	0.80±1.44	0.22±0.43
No. of fetuses affected (%)	5 (2.4)	15 (8.0)	16 (6.8)	4 (1.7)
Cranial abnormalities[a]	0.00±0.00	0.00±0.00	0.15±0.67	0.00±0.00
Exencephaly (%)	0 (0.0)	0 (0.0)	3 (1.3)	0 (0.0)
Trunk abnormalities[a]	0.26±0.45	0.79±1.32	0.65±2.21	0.22±0.43
Edema and hematoma (%)	5 (2.4)	15 (8.0)	13 (5.5)	4 (1.7)

[a]Indicates mean number of fetuses affected per litter.

Table 3
Visceral Abnormalities of Fetuses From Dams Orally Administered With Korean Red Ginseng Extract (KRGE)

Treatment (mg/kg/day)	Vehicle	KRGE (20)	KRGE (200)	KRGE (2,000)
No. of dams used	17	16	17	17
No. of fetuses examined/litter	5.94±1.03	5.50±1.05	5.67±1033	6.35±1.64
Total no. of fetuses examined	101	88	102	108
Abnormalities observed[a]	1.35±1.05	1.13±0.67	2.00±1.86	1.06±0.88
No. of fetuses affected (%)	22 (21.8)	13 (14.8)	27 (26.5)	15 (13.9)
Brain abnormalities[a]	0.00±0.00	0.00±0.00	0.06±0.24	0.00±0.00
Misshapen cerebrum (%)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)
Oral abnormalities[a]	0.24±0.56	0.00±0.00	0.11±0.32	0.00±0.00
Cleft palate (%)	4 (4.0)	0 (0.0)	2 (2.0)	0 (0.0)
Thymic abnormalities[a]	0.12±0.33	0.44±0.51	0.33±0.49	0.12±0.33
Remnant in the neck (%)	2 (2.0)	5 (5.7)	4 (3.9)	1 (0.9)
Enlarged thymus (%)	0 (0.0)	2 (2.3)	1 (1.0)	1 (0.9)
Small thymus (%)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)
Renal abnormalities[a]	1.00±0.94	0.69±0.70	1.44±1.46	0.94±0.83
Dilatated pelvis (%)	10 (9.9)	6 (6.8)	18 (17.6)	14 (13.0)
Malpositioned kidneys (%)	7 (6.9)	4 (4.5)	5 (4.9)	2 (1.9)
Small kidneys (%)	0 (0.0)	1 (1.1)	3 (2.9)	0 (0.0)
Ocular abnormalities[a]	0.00±0.00	0.00±0.00	0.33±0.49	0.00±0.00
Anophthalmia (%)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)

[a]Indicates mean number of fetuses affected per litter.

Table 4
Skeletal Abnormalities of Fetuses From Dams Orally Administered With Korean Red Ginseng Extract (KRGE)

Treatment (mg/kg/day)	Vehicle	KRGE (20)	KRGE (200)	KRGE (2,000)
No. of dams used	17	16	17	17
No. of fetuses examined/litter	6.12±1.25	4.94 ±1.85	6.24±1.54	6.76±1.33
Total no. of fetuses examined	104	79	106	115
Abnormalities observed[a]	2.06±1.83	2.13±1.76	3.06±2.51	2.00±2.06
No. of fetuses affected (%)	35 (33.7)	34 (43.0)	52 (49.1)	34 (29.6)
Cranial abnormalities[a]	0.12±0.28	0.12±0.35	0.06±0.24	0.00±0.00
Incomplete ossification (%)	2 (1.9)	2 (2.5)	1 (0.9)	0 (0.0)
Vertebral abnormalities[a]	0.00±0.00	0.00±0.00	0.06±0.24	0.06±0.24
Fused vertebra (%)	0 (0.0)	0 (0.0)	1 (0.9)	0 (0.0)
Cleaved vertebra (%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.9)
Costal abnormalities[a]	0.29±0.47	0.50±0.73	1.00±1.37[b]	0.59±0.94
Supernumerary ribs (%)	5 (4.8)	8 (10.1)	16 (15.1)	9 (7.8)
Misaligned ribs (%)	0 (0.0)	0 (0.0)	1 (0.9)	1 (0.9)
Sternal abnormalities[a]	1.65±1.52	1.50±1.45	2.59±2.03	1.35±1.39
Incomplete ossification (%)	28 (26.9)	24 (30.4)	44 (41.5)	23 (20.0)

[a]Indicates mean number of fetuses affected per litter.
[b]Significantly different from vehicle control ($P < 0.05$).

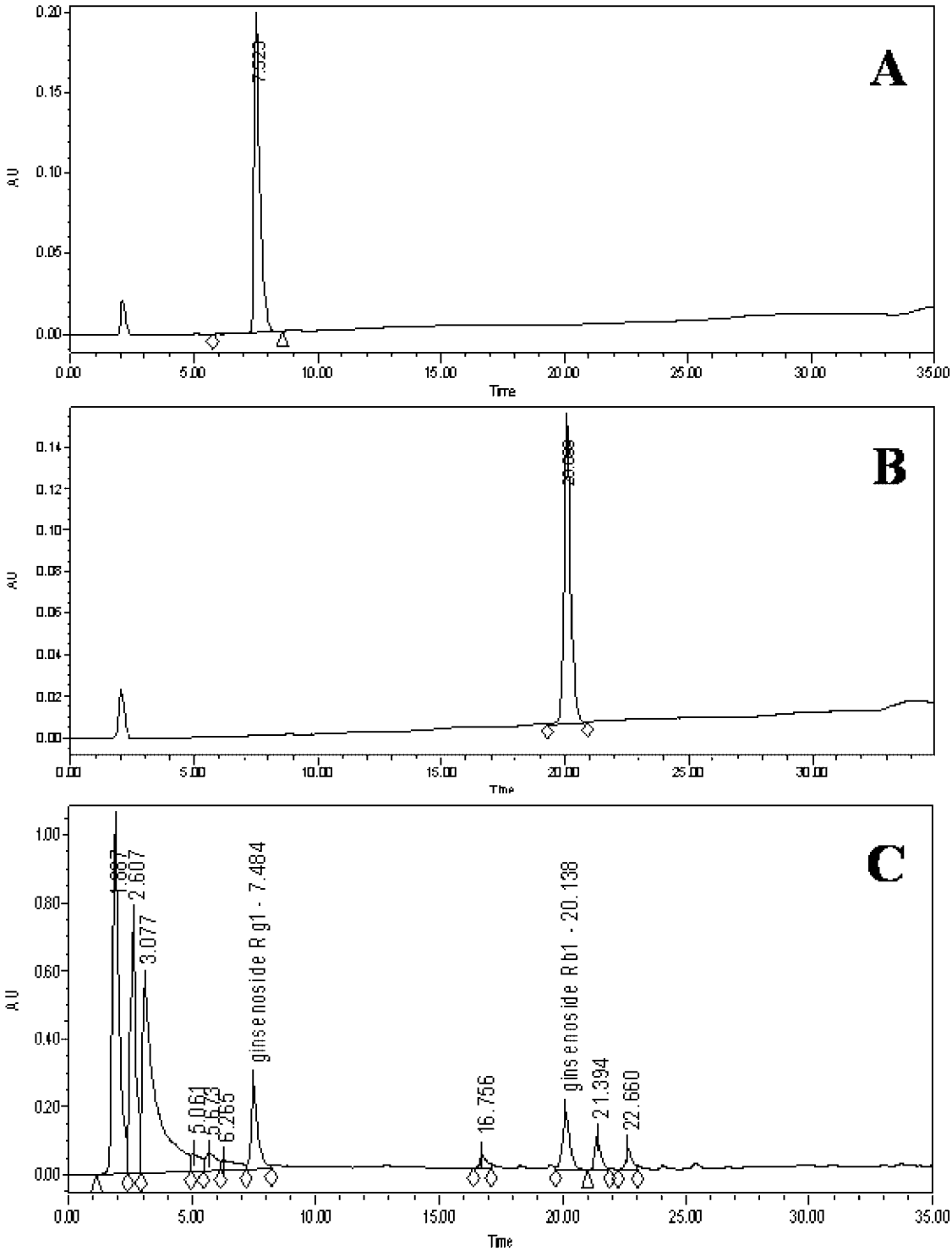

Fig. 2. HPLC chromatograms of standard Rg1 (**A**), Rb1 (**B**), and components of KRGE including Rg1 and Rb1 (**C**). The retention times for Rg1 and Rb1 were 7.5 and 20.1 min, respectively.

necessary before a conclusion about safety issues can be reached.

In this study, the KRGE was prepared according to the standard procedures in Oriental medicine, and orally applied to mice from 2 weeks before mating to the end stage of gestation. Since there are usually dosage limitations in human studies, we administered KRGE up to an extremely high dose, i.e., 2,000 mg/kg/day (≈200 times clinical doses), the upper-limit dose recommended by the KFDA. We evaluated all possible parameters of maternal reproductive and embryo-fetal toxicities including external, visceral, and skeletal

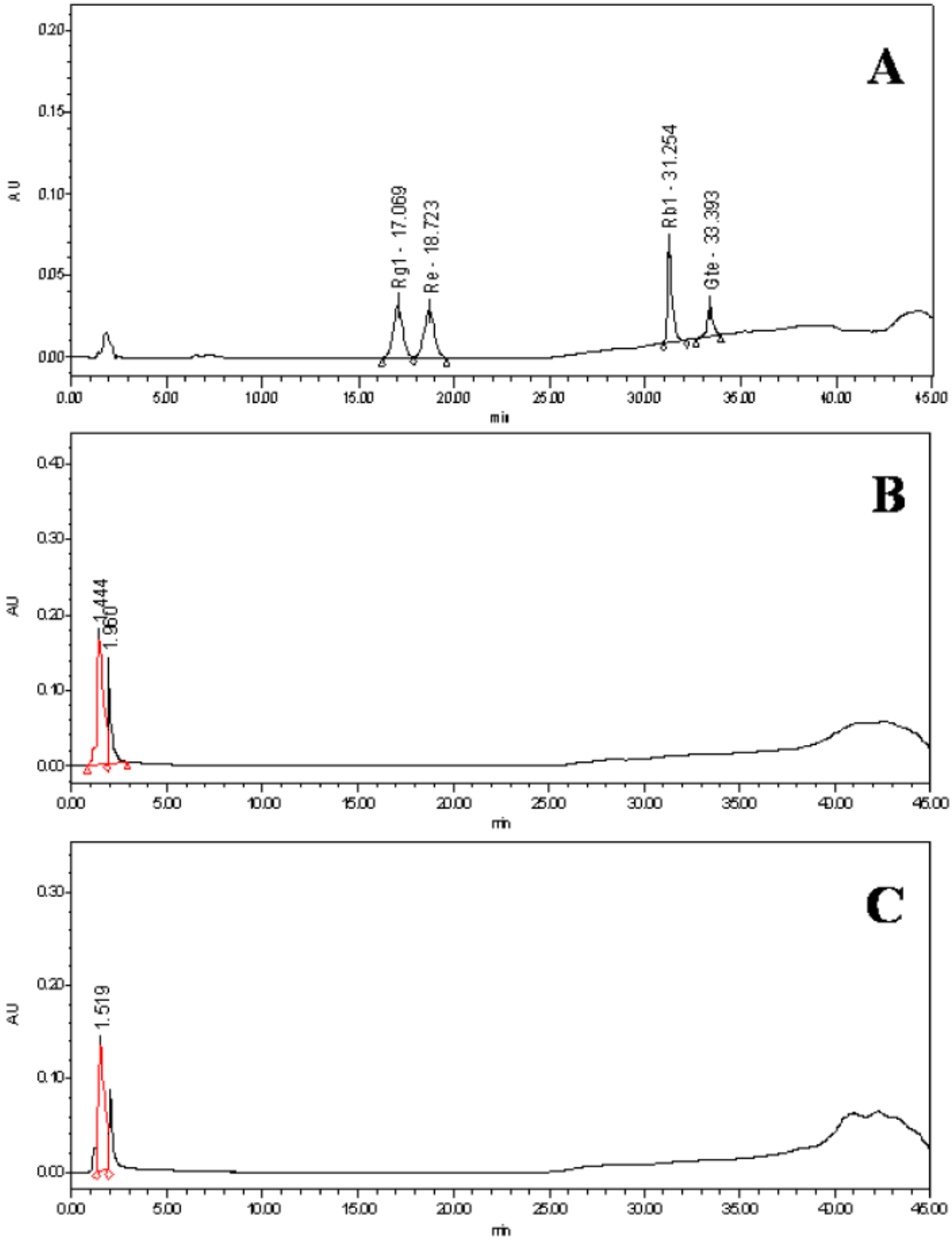

Fig. 3. A: HPLC chromatograms of ginsenosides from sera after spiking of a standard mixture containing Rg1, Rb1, Re, and ginseng total extract (Gte). **B:** HPLC profile of an animal 30 min after administration with KRGE (2,000 mg/kg/day). **C:** HPLC profile of an animal 2 h after administration with KRGE (2,000 mg/kg/day). No ginsenosides were detected from the sera of mice orally administered with KRGE.

abnormalities. KRGE influenced neither the embryonic implantation nor fetal growth. Although hematoma and edema, renal pelvic dilatation, and sternal incomplete ossification tended to increase in the groups treated with low (20 mg/kg/day) or medium doses (200 mg/kg/day) of KRGE, no dose-response effects and statistical significance were observed. Notably, 200 mg/kg/day (≈20 times clinical doses) of KRGE appeared to

significantly increase the incidences of supernumerary (14th) ribs in the fetuses. However, a higher dose (2,000 mg/kg/day) did not increase the rudimentary ribs, which are not considered to cause functional impairment of the body.

In our previous study, KRGE did not cause any adverse effects on embryonic implantation, fetal growth and delivery, and postnatal physical development and reproductive function (Kim et al., 2006). Therefore, there was no convincing evidence that KRGE administration would cause embryo-fetal toxicity in mice. In contrast to previous in vitro findings that suggested ginseng can be teratogenic (Chan et al., 2003, 2004; Liu et al., 2005, 2006), our results are in agreement with previous reports that showed the use of herbal medicine including ginseng did not cause abnormalities during pregnancy in human (Holst et al., 2008; Seely et al., 2008), suggesting that administration of ginseng has no adverse effects in vivo when used in the range of a maximum-tolerated dose.

To further verify that there were no adverse effects in vivo, we measured the concentrations of Rb1, Rg1, and Re, the ginsenosides reported to be teratogenic in the in vitro studies (Chan et al., 2003, 2004; Liu et al., 2005, 2006), in serum samples following KRGE administration. Contrary to the results of the ingredient analysis, there was no evidence of Rb1, Rg1, and Re in mouse sera, suggesting that these ginsenosides do not reach a detectable blood level by oral treatment of KRGE up to the upper-limit dose ($\approx$200 times clinical doses). It is well known that protopanaxadiols (including Rb1) and protopanaxatriols (including Re and Rg1) are metabolized mainly to compound-K and Rh1, respectively, by enzymes such as β-$_D$-glucosidase from intestinal bacteria (Akao et al., 1998; Hasegawa et al., 1996; Tawab et al., 2003), resulting in the absorption of minimal portions of Rb1 (0.1%) and Rg1 (1.9%) from the gastrointestinal (GI) tract (Takino, 1994). Since the concentrations of Rb1 and Rg1 in KRGE were 0.61 and 0.9%, their blood levels following administration of the highest dose (2,000 mg/kg/day) of KRGE were estimated to be 12 and 34.2 ng/ml, respectively. The blood concentrations of Rb1 and Rg1 might be much lower than their minimal embryotoxic concentrations in vitro; 30 and 10 μg/ml for Rb1 and Rg1, respectively (Chan et al., 2003; Liu et al., 2005, 2006). Furthermore, the maximum blood concentrations of intact ginsenosides were very low, not higher than 20 ng/ml, in human studies (Ji et al., 2004; Tawab et al., 2003), although decomposition rates of the ginsenosides might vary between animal species and humans.

In the present study, no considerable adverse effects of KRGE on the embryonic implantation and fetal development were observed. These results were in contrast to the teratogenic affects of major ginsenosides reported in the in vitro studies. Such different results may be due to the decomposition of the ginsenosides to diverse pharmacologically active or inactive metabolites in the GI tract (Kang et al., 2002). Therefore, it is suggested that oral administration of KRGE prepared according to the standard procedures in Oriental medicine does not cause abnormalities in embryo-fetal development in mice, and that further studies on the parenteral injection of ginsenosides, which were teratogenic in vitro experiments, or their metabolites into animals will be required to better understand the embryotoxic mechanisms of ginsenosides.

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