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Research Article

KRG and its major ginsenosides do not show distinct steroidogenic activities examined by the OECD test guideline 440 and 456 assays

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ABSTRACT

Background: Ginseng has been used as a traditional medicine for treatment of many diseases and for general health maintenance. Previously, we showed that ginseng did not demonstrate estrogenic property in ovariectomized mouse model. However, it is still possible that disruption of steroidogenesis leading to indirect hormonal activity.
Methods: The hormonal activities were examined in compliance with OECD guidelines for detecting endocrine disrupting chemicals: test guideline (TG) No. 456 (an *in vitro* assay method for detecting steroidogenesis property) and TG No. 440 (an *in vivo* short-term screening method for chemicals with uterotrophic property).
Results: Korean Red Ginseng (KRG) and ginsenosides Rb1, Rg1, and Rg3 did not interfere with estrogen and testosterone hormone synthesis as examined in H295 cells according to TG 456. KRG treatment to ovariectomized mice did not show a significant change in uterine weight. In addition, serum estrogen and testosterone levels were not change by KRG intake.
Conclusion: These results clearly demonstrate that there is no steroidogenic activity associated with KRG and no disruption of the hypothalamic-pituitary-gonadal axis by KRG. Additional tests will be performed in pursuit of cellular molecular targets of ginseng to manifest mode of action.

1. Introduction

Ginseng has been used to improve the physical strength and immune function [1–5]. Numerous ginseng components such as peptides, fatty acids, polyacetylenic alcohols, polysaccharides, and ginsenosides have been characterized [6–11]. Ginsenoside-Rb1 (Rb1) and ginsenoside-Rg1 (Rg1) are present in ginseng root, and Korean Red Ginseng (KRG) contains ginsenoside-Rg3 (Rg3). In addition to various physiological functions, the antitumor effect of ginseng has been proved through various experiments [12,13].

Beneficial effects of KRG on menopausal symptoms were first reported in 1999 by a Japanese group [14]. Since then, double-blind, five randomized, placebo-controlled clinical trials were conducted showing some beneficial effects on middle-aged menopausal women when supplemented for 4 to 12 weeks at a dosage of 1-3 g/day [14–20]. These effects are probably due to various valuable functions of KRG such as adaptogenic effect, improvement of blood circulation, immune-modulatory, strong cognitive and anti-oxidative effects [21–25]. However, cellular targets or the mode of action for these effects were not clearly identified. It has been hypothesized that ginsenosides function as partial agonists, antagonists, or receptor agonists depending on the microenvironment [26]. It has been suggested that KRG may influence hormonal activity such as estrogen, androgen, and glucocorticoid [27,28]. We and others have shown *in vitro* data displaying such activity and our data showed that *in vitro* activity was observed only at high doses and *in vivo* estrogenic activity was not detected [29]. Few data showed such *in vitro* activity leading to *in vivo* function. It is important to clarify no estrogenic activity associated with KRG *in vivo*. To this end, we aimed to examine steroidogenic activity of KRG and its major ginsenosides in this study because disruption of steroidogenesis can affect hormone activity indirectly. As far as we

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know, no study yet have examined steroidogenic hormone synthetic activity of ginseng.

The Organization for Economic Co-operation and Development (OECD) has developed international standardization methods to evaluate endocrine disrupting chemicals (EDCs). EDCs are generally defined as substances in our environment, food and consumer products that can disrupt the hormonal balance in humans and wildlife and result in adverse health effects [30]. In 1997, the OECD began developing guidelines for the assessment of EDCs, managed by the Endocrine Disruptors Testing and Assessment task force [30,31]. The OECD updated their conceptual framework for assessment of EDCs in 2012 [32]. This document provides a five-step guideline for identifying EDCs from level 1 to 5. It describes in vitro test methods, including a cell-based transcriptional activation assay at level 2 and *in vivo* testing methods in animals at level 3. Level 4 and 5 contain methods for multi-generational effect. Level 2 test guidelines can evaluate EDCs affecting estrogen, androgen, and thyroid hormone activities and steroidogenesis [31,33]. These very detailed and standardized protocols are applied in this study to evaluate estrogenic and steroidogenic properties of KRG and selected ginsenosides. The uterotrophic and steroidogenic activity that may disturb *in vivo* hormonal synthesis was assessed using test guideline (TG) 456 and 440, in accordance with the OECD guidelines for chemical testing [34,35]. TG 456 generally detects substances that inhibit or induce enzyme activity in the steroid synthesis pathway [36]. TG440 is uterotrophic bioassay for *in vivo* short-term screening of estrogenic properties. It is based on the increase in uterotrophic response or uterine weight. The uterotrophic bioassay relies on an animal test system for its sensitivity, independent of the hypothalamic-pituitary-ovarian axis.

2. Materials and methods

2.1. Materials

KRG and ginsenosides were supplied by the Korea Ginseng Corporation (Daejeon, Republic of Korea). Dimethyl sulfoxide (DMSO), forskolin and prochloraz were purchased from Sigma Aldrich (St. Louis, MO, USA). 5L79 diet were purchased from Charles River (Wilmington, MA, USA). The diet was composed crude protein (> 18.0%), crude fat (> 5.0%), crude fiber (< 5.0%), ash (< 8.0%), calcium (> 0.5 %), and phosphorus (> 0.4%). Forskolin and prochloraz were dissolved in DMSO.

2.2. Cell culture and reagents

H295R cells were cultured in DMEM/F12 (Welgene, Gyeongsan-si, Republic of Korea) supplemented with 1% ITS + premix supplement and 2.5% Nu-serum (Corning, NY, USA). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and they were sub-cultured when cell confluence reached 80%.

2.3. Steroidogenesis assay

Steroidogenesis was measured using human adreno-carcinoma cells H295R and the assessment was performed in compliance with OECD TG456 (OECD, 2011). Forskolin was supplemented at a concentration of 10 μM as a positive control, and ginsenosides and KRG were added at a concentration as indicated in Fig. 1. In brief, 3×10^5 cells/1 ml/well were seeded on to a 24 well microplate and treatment was performed after 24 h of seeding. After 48 h of treatment,

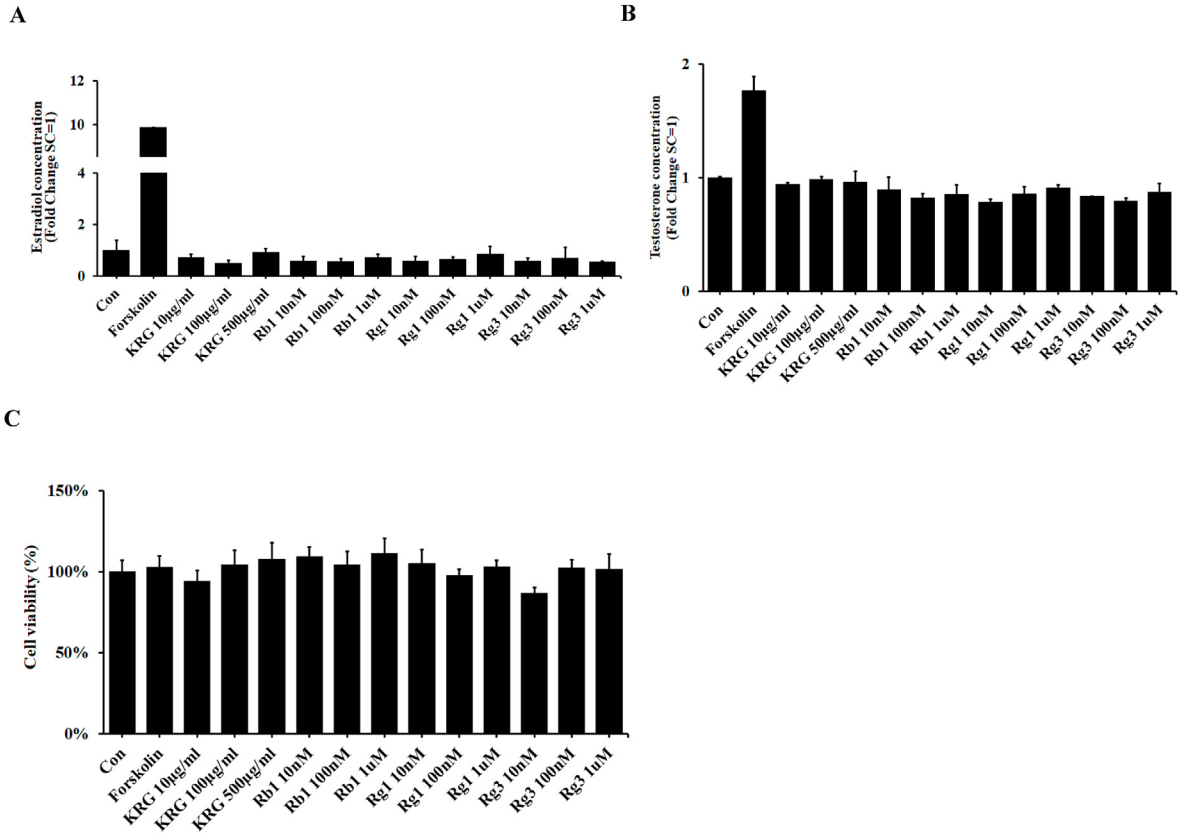

Fig. 1. Effect of chemicals on steroid hormone production using OECD test guideline No. 456. H295R cells were treated for 48 h with 10 μM forskolin or KRG (10 μg/ml to 500 μg/ml), (B) Rg1 (1.00×10^{-8} to 1.00×10^{-6} M), Rg3 (1.00×10^{-8} to 1.00×10^{-6} M), Rb1 (1.00×10^{-8} to 1.00×10^{-6} M). After treatment, estrogen (A), testosterone (B) and cell viability (C) were measured.

hormones in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA) according to the protocol of Cayman's ELISA kit (Ann Arbor, MI, USA).

2.4. Mice

Female Slc:ICR mice (6-week old) were purchased from ORIENT (Gapyeong, Republic of Korea). All procedures were performed according to the recommendations for the proper use and approved protocols and care of animals at the specific pathogen-free housing facility at Konkuk University. Mouse studies were approved by the Konkuk University Institutional Animal Care and Use Committee (Ref. No.:KU21012).

2.5. Mouse model for uterotrophic assay

The uterine hypertrophy mouse model was developed according to the schedule as shown in Fig. 2A. For ovariectomy (OVX), mice were anesthetized and then the abdominal cavity was opened. The ovaries were removed from both ends of the fallopian tube. To determine the uterine hypertrophy effect of KRG, mice were randomly allocated to the following 6 groups (n = 6/group): (1) sham control (without OVX), (2) OVX, (3) OVX + E2 (1 μg/kg), (4) OVX + KRG (200 mg/kg), (5) OVX + KRG (400 mg/kg), (6) OVX + KRG (800 mg/kg). The Korean Herbal Pharmacopoeia recommends a daily KRG intake of 1.5 - 10 g for medicinal purposes, which can be increased up to 30 g [37]. In general, 3 g/day is the recommended daily dosage. The dose of KRG is 500 mg/kg when calculated from 30 g/60 kg/day. However, using animal to human equivalent dose based on body surface, 3 g for 60 kg man dosage is equivalent to 615 mg/kg KRG dosage to mouse. Therefore, to be conservative, we used 800 mg/kg KRG. Oral dosage of KRG used in mouse studies varied for different model systems but were usually within the range of 50-200 mg/kg for testing the effect and function of KRG. Therefore, we confirmed the effect from the low concentration of 200 mg/kg to the high concentration of 800 mg/kg. Seven days after OVX, sesame oil containing E2 was continuously injected s.c. daily and KRG was administered orally. Body weight, feeding activity, and clinical symptoms were checked to confirm the animal's condition as specified by OECD test guideline 440(Supplementary Table). KRG was administered orally every day, and the condition and feeding activity of mice were monitored. Mice were sacrificed on day 16. The uterine weight was confirmed by sacrificing the mice, and there was no difference between the wet and blotted uterine weights. The uterine tissues were fixed in 4% normal buffer formaldehyde for histological analysis using hematoxylin and eosin (H&E) staining. Hormones in the serum were measured by ELISA according to the protocol of Cayman's ELISA kit (Ann Arbor, MI, USA).

2.6. Statistical analysis

All data were analyzed using GraphPad Prism 7.0 (Graph Pad Software, La Jolla, CA, USA). The data were expressed as mean ± SD and statistical analysis of the data was performed by 2-tailed Student's t-test and $p < 0.05$ (*) was considered to indicate statistical significance. All *in vitro* experiments were performed in triplicates and repeated at least three times.

3. Results

3.1. Measurement of steroidogenesis

We used the TG No. 456 (an *in vitro* assay method for detecting the steroidogenesis property) assay to detect induction of E2 or testosterone. All experiments strictly followed the guidelines. As

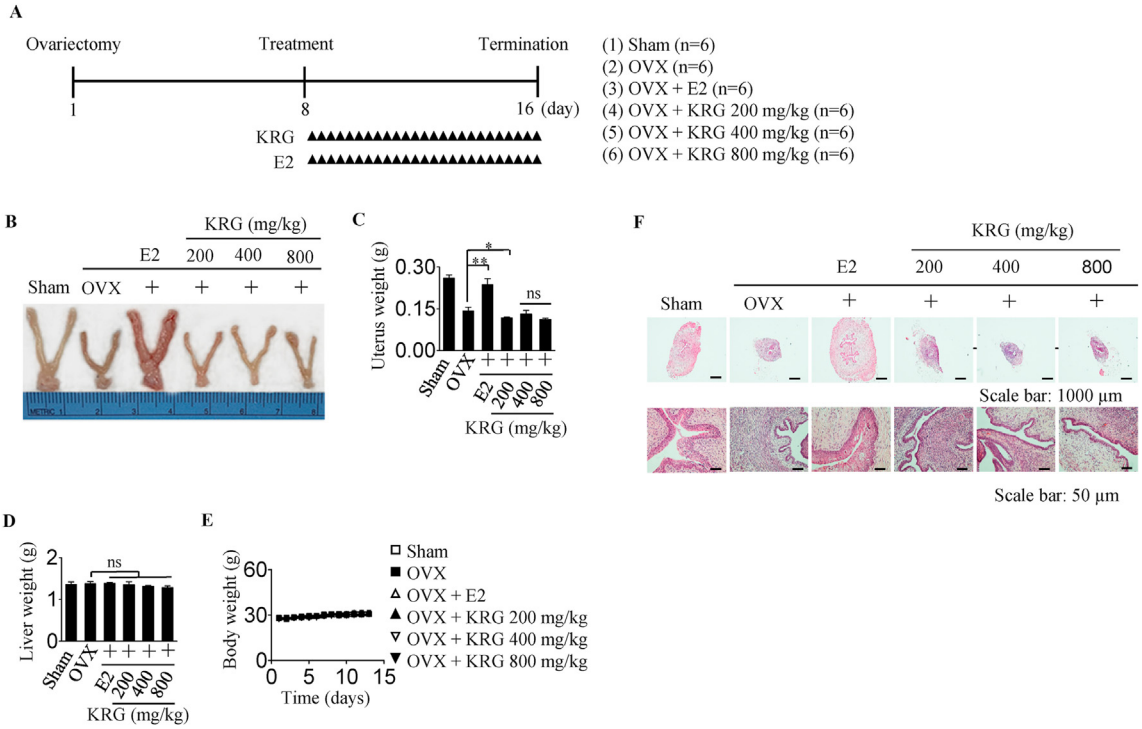

Fig. 2. Uterotrophic response of KRG treated in the OVX model using OECD test guideline No. 440. (A) Experimental schedule for KRG and E2 treatment. (B) Images of treated uterus. (C) Uterus weight (*$P < 0.05$. **$P < 0.01$, ns : no significant). (D) Liver weight (E) Change of body weight during treatment. (F) H&E stained uterine tissues.

shown in Fig. 1, forskolin was used as a positive control for E2 and testosterone. All experiments fulfilled the guideline that the fold change value of 10 μM forskolin should be 7.5 times that of control in E2 and 1.5 times that of testosterone [34]. The fold change values of KRG, Rb1, Rg1, and Rg3 were 0.9, 0.7, 0.8, and 0.5, respectively compared to the vehicle treated control. KRG, Rb1, Rg1, and Rg3 did not interfere with estrogen and testosterone hormone production (Fig. 1A and B). There was no change in cell viability (Fig. 1C).

3.2. Uterotrophic response of KRG

The uterine hypertrophy mouse model was developed according to the schedule as shown in Fig. 2A. While the uterotrophic response in the OVX + E2 group was increased, the OVX (negative control) and OVX + KRG treatment groups did not show a significant increase (Fig. 2B). The weight of the uterus in the OVX + KRG treatment groups showed a similar result as the uterotrophic response (Fig. 2C). Although the uterus weight was changed, the liver weight in all experiment mice group showed no difference (Fig. 2D) and the body weight was consistently maintained (Fig. 2E), indicating that KRG administration caused no overt toxicities related to treatment. Compared to the OVX group, the intima (secretory glands) was significantly increased in the E2 treatment group due to luminal epithelium proliferation. There was no significant change in histological findings between KRG administration groups of increasing dosages. In histological analysis, proliferation of the uterus and fallopian tubes was observed in the OVX + E2 group compared to atrophy of uterus and fallopian tubes in the OVX group (Fig. 2F). The serum E2 and testosterone levels in the OVX group were not changed by KRG treatment (Fig. 3A and B).

4. Discussion

Ginseng is one of the world's best-selling herbs for use in complementary and alternative medicine due to its beneficial effects on cognition and blood circulation, as well as its anti-aging, anti-cancer, and anti-diabetic effects [38–41]. Ginsenosides are the pharmacological constituents responsible for the beneficial effects of ginseng. Identification of the mechanism by which ginseng affects diverse signaling pathways is essential for the development of multi-target phytomedicine for various diseases [42]. Research studies have shown that nuclear hormone receptors are cellular targets of ginsenosides under different *in vitro* conditions [27] but rigorous and standardized results are necessary to demonstrate the detailed mode of action. Extensive review of the literature reveals that direct binding or measurement of ginsenoside-occupied receptor was not found. It is very necessary and important to obtain extensive and thorough experimental data using standardized methods in the course of development of functional targets and application of ginseng usage.

KRG, Rb1, Rg1, and Rg3 have no associated steroidogenesis activity. In addition, our preliminary data showed that KRG does not evoke or aggravate breast tumor induced by E2 in an orthotopic breast cancer model (Supplement. 1). Chemicals can interfere with the endocrine system in other ways than through the hormone receptor, such as effects on the hypothalamic-pituitary-gonadal axis (HPG) that can only be detected in animal studies such as changes in hormone levels. Our data showed that KRG did not interfere with the sex steroid hormone levels in 440 ovariectomized mouse model systems, which indicates that it is unlikely that KRG might interfere with the HPG axis. Phytoestrogens are similar in chemical structure to the mammalian estrogen and bind to estrogen receptors alpha and beta may be with a preference for the estrogen receptor beta [43–45]. The well-studied phytoestrogen genistein showed a weak binding affinity to ER *in vitro* with positive uterotrophic assay at high doses [46]. The NOAEL of genistein from rodents is considered to be approximately 2.5-50 mg/kg/day based on the hormonally induced changes [46]. However, our previous and the data presented here did not show *in vivo* estrogenic response and elicit any changes in estrogen and testosterone synthesis. On comparing with these data, it may be not adequate to consider KRG as one member of the phytoestrogen based on the current available data. The reported beneficial activity in sex-associated clinical trials [14–20] and the *in vivo* animal models of KRG are very likely to be mediated by other cellular pathways [47]. More rigorous study is needed to show the mode of action for the reported clinical effects of KRG on menopause.

The OECD provides a flow chart and all possible scenarios for holistic evaluation of data to assess whether a substance should be regarded as an EDC [30,32]. TG440 *in vivo* screening assay at Level 3 is designed to provide a yes/no (qualitative) answer about the ability to interact with the estrogen receptor or interfere with steroidogenesis [35]. Level 3 assays are originally designed primarily for revealing the mechanisms of action (MOA) and hazard detection, and they may be used for making regulatory decisions in some circumstances [31]. They are designed of deliberately high to provide some information about the potency of a chemical *in vivo*, therefore negative 440 of KRG indicates that the ER associated

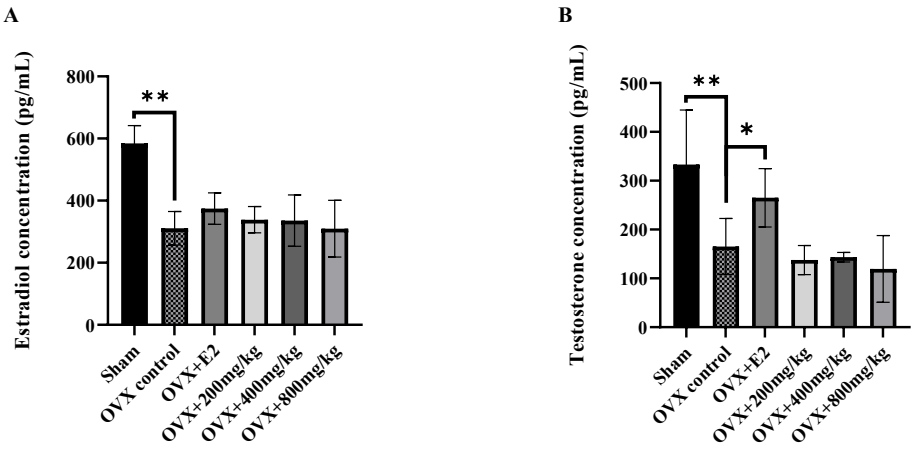

Fig. 3. Estrogen and testosterone serum levels of KRG treated in the OVX mouse model. Serum levels of estradiol (A) and testosterone (B). All experiments were repeated at least three times (*$P < 0.05$. **$P < 0.01$).

concerns are of the lowest level. In addition, our TG456 data showed no disruption with steroidogenesis. Continuation of our research for identifying the MOA for KRG for its beneficial effect on menopause will reveal and provide the MOA for its beneficial health-promoting effects in middle-aged women.

Declaration of competing interest

The authors declared that they had no conflicts of interests.

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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.2022.09.002.

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