Korean Red Ginseng alleviates dehydroepiandrosterone-induced polycystic ovarian syndrome in rats via its antiinflammatory and antioxidant activities

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ABSTRACT

Background: Beneficial effects of Korean Red Ginseng (KRG) on polycystic ovarian syndrome (PCOS) remains unclear.

Methods: We examined whether pretreatment (daily from 2 hours before PCOS induction) with KRG extract in water (KRGE; 75 and 150 mg/kg/day, p.o.) could exert a favorable effect in a dehydroepiandrosterone (DHEA)-induced PCOS rat model.

Results: Pretreatment with KRGE significantly inhibited the elevation of body and ovary weights, the increase in number and size of ovarian cysts, and the elevation of serum testosterone and estradiol levels induced by DHEA. Pretreatment with KRGE also inhibited macrophage infiltration and enhanced mRNA expression levels of chemokines [interleukin (IL)-8, monocyte chemoattractant protein-1], proinflammatory cytokines (IL-1β, IL-6), and inducible nitric oxide synthase in ovaries induced by DHEA. It also prevented the reduction in mRNA expression of growth factors (epidermal growth factor, transforming growth factor-beta (EGF, TGF-β)) related to inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cell pathway and stimulation of the nuclear factor erythroid 2-related factor 2 pathway. Interestingly, KRGE or representative ginsenosides (Rb1, Rg1, and Rg3(s)) inhibited the activity of inflammatory enzymes cyclooxygenase-2 and iNOS, cytosolic p-IkB, and nuclear p–nuclear factor kappa-light-chain-enhancer of activated B cell pathway and stimulation of the nuclear factor erythroid–derived 2-related factor 2 pathway. Interestingly, KRGE or representative ginsenosides (Rb1, Rg1, and Rg3(s)) inhibited the activity of inflammatory enzymes cyclooxygenase-2 and iNOS, cytosolic p-IkB, and nuclear p–nuclear factor kappa-light-chain-enhancer of activated B cell pathway and stimulation of the nuclear factor erythroid–derived 2-related factor 2 pathway. Interestingly, KRGE or representative ginsenosides (Rb1, Rg1, and Rg3(s)) inhibited the activity of inflammatory enzymes cyclooxygenase-2 and iNOS, cytosolic p-IkB, and nuclear p–nuclear factor kappa-light-chain-enhancer of activated B cell pathway and stimulation of the nuclear factor erythroid–derived 2-related factor 2 pathway. 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Conclusion: These results provide that KRGE could prevent DHEA-induced PCOS via antiinflammatory and antioxidant activities. Thus, KRGE may be used in preventive and therapeutic strategies for PCOS-like symptoms.
1. Introduction

Polycystic ovary syndrome (PCOS) is the most common cause of female subfertility, affecting approximately 12% of women during and beyond their reproductive years [1,2]. Its main features include chronic oligo-ovulation or anovulation, impaired fertility, and metabolic syndromes related to type II diabetes mellitus, hyperinsulinemia, hyperandrogenism (clinical or biochemical), hirsutism, acne, increased incidence of endometrial cancer, and polycystic ovaries (histopathological) [1,3–5]. Although genetic factors and environmental factors such as diet and stress might be associated to the development of PCOS [6], the pathogenesis of PCOS remains unclear.

Pharmacological agents for treatment of PCOS are very limited. Clomiphene citrate is a standard treatment to produce ovulation in women with PCOS. It is still considered the first line drug [7]. However, clomiphene citrate has some adverse effects, including high failure rate of ovulation (25–30%), significant discrepancy between ovulation and pregnancy rates, antiestrogenic actions on the endometrium and cervical mucous, long half-life, and multi-follicular ovulation. In addition, clomiphene citrate is expensive and has some adverse effects such as neural tube defects [8,9].

Complementary and alternative medicines such as herbs, phytochemicals, and dietary supplements can mitigate PCOS or decrease the disadvantages of chemicals such as clomiphene citrate and metformin used for PCOS [10]. However, there are minimal evidences that complementary and alternative medicines are safe and efficacious. Thus, new preventive and therapeutic strategies such as complementary and alternative treatment should be evaluated to alleviate PCOS, regulate hormones, and improve quality of life of PCOS women. Panax (P.) ginseng (Korean ginseng) is a popular herbal medicine that has been widely used for more than 2,000 years in Oriental countries. It has long been considered as “the king of herbs” with various pharmacological activities on the immune, circulatory, respiratory, endocrine, gastrointestinal, and central nervous systems [11–13]. P. ginseng may reduce menopausal symptoms such as sleep disorder, depression, and anxiety. It is often used as a natural estrogen replacement therapy. Furthermore, P. ginseng extract can activate the growth of estrogen receptor (ER) positive (+) cells in vitro. Ginsenoside Rb1 and Rg1 can stimulate ERs with estrogen-like activity [14–16]. In ovariectomized mice, P. ginseng can recover the estrus cycle and exert significant estrogenic effects as suggested by the reversal of atrophy of the uterus and vagina with upregulated expression of ER α and ER β in reproductive tissues. Meanwhile, P. ginseng can significantly increase serum estradiol and reduced follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels in the serum [17]. These reports highlight its possible use for PCOS. Recently, we introduced that Korean Red Ginseng extract in water (KRGE) can reduce the formation of antral follicles and increase the number of corpora lutea in polycystic ovaries associated with normalization of nerve growth factor in an estradiol valerate–treated rat model for PCOS [18]. However, the effect of KRGE on PCOS has not been clearly elaborated yet. Therefore, the objective of this study was to determine the preventive and therapeutic potential of KRGE for PCOS using a dehydroepiandrosterone (DHEA)-mediated rat model and examine the role of its anti-inflammatory and antioxidant activities in this regard.

2. Materials and methods

2.1. Animals and ethical statement

Female Sprague Dawley rats (42 days old; Naribioct Co., Ltd., Seoul, Korea) were kept at a constant temperature of 23 ± 3 °C with a 12-hours (hrs) light-dark cycle (lights on from 08:00 to 20:00) and fed food and water ad libitum. The animals were allowed to habituate to the housing facilities for 1 week before the experiments. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Kyung Hee University. In this process, proper randomization of laboratory animals and handling of data were performed in a blind manner in accordance with recent recommendations from an NIH workshop on preclinical models of neurological diseases [6].

2.2. Preparation of KRGE

KRGE (Korea Ginseng Corporation, Daejeon, Korea) was prepared, as previously described [19], from the roots of 6-year-old fresh P. ginseng Meyer. KRGE contained major ginsenosides Rb1 (7.44 mg/g), Rb2 (2.59 mg/g), Rc (3.04 mg/g), Rd (0.91 mg/g), Re (1.86 mg/g), Rf (1.24 mg/g), Rg1 (1.79 mg/g), Rg2s (1.24 mg/g), Rg3s (1.39 mg/g), and Rb1 (1.01 mg/g) and other minor ginsenosides, as determined by high-performance liquid chromatography.

2.3. PCOS induction and KRGE treatment

Female 42-day-old postpubertal rats were divided into an experimental group for pretreatment with KRGE. This group was then divided into five subgroups (n = 9 per group): (1) sham group [saline, subcutaneous (s.c.) + saline, p.o.], (2) DHEA group [DHEA, 60 mg/kg body weight (BW)/day, s.c. + saline, p.o.], (3) DHEA + KRGE 75 group [DHEA, 60 mg/kg BW/day, s.c. + KRGE 75 mg/kg BW/day, p.o.], (4) DHEA + KRGE 150 group [DHEA, 60 mg/kg BW/day, s.c. + KRGE 150 mg/kg BW/day, p.o.], and (5) KRGE group [saline, s.c. + KRGE 150 mg/kg BW/day, p.o.]. The PCOS model was induced by treatment with DHEA as described previously [20] with some modifications [12]. To examine the preventive effects of KRGE in PCOS, KRGE was given orally daily for 20 consecutive days starting 2 hrs before the first DHEA treatment. Rats in the sham or KRGE group were treated with saline or KRGE without DHEA treatment.

2.4. BW and vaginal smears

BW was measured before treatment with DHEA, KRGE, or saline daily during the experimental period. The stage of estrous cycle was also measured by microscopic observation of the predominant cell type in vaginal smears obtained daily beginning on Day 0 in KRGE-pretreated rats and on Day 25 (48 days after birth) in KRGE-pretreated rats as previously described [21,22].

2.5. Blood serum preparation and examination of hormone levels

Serum was prepared as described previously [22]. Serum testosterone and estradiol levels were examined using enzyme-linked immunosorbent serologic assay (ELISA). Ultrasensitive ELISA Kits for rat testosterone (Mercodia, Uppsala, Sweden), rat estradiol (Mercodia), rat LH (Enzo Life Sciences, Farmingdale, NY), and rat FSH (Cusabio biotech, Houston, TX) were used according to the manufacturer’s instructions.

2.6. Histological examination

Twenty days after DHEA treatment, ovaries were sampled, and paraffin sections (5 μm in thickness) were prepared. After
performing hematoxylin–eosin staining, the number of cysts was counted as previously described [22].

2.7. Detection of apoptosis with the terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using an ApoTag Peroxidase in situ Apoptosis Detection Kit (S7100) (Millipore, Burlington, MA) according to the manufacturer’s instructions and as previously described [23].

2.8. Immunohistological analysis

The paraffin sections were performed by immunohistochemistry as previously described [22]. Briefly, the sections were incubated with rabbit antiantibodies against calcium binding molecule (Iba)-1 (1:1,500; Wako Pure Chemical, Osaka, Japan), followed by biotinylated mouse/rabbit IgG antibody (1:1,500; Vector Laboratories, Burlingame, CA) and with avidin–biotinylated horseradish peroxidase (1:250; Vector Laboratories).

2.9. Immunoblot analysis

Twenty days after the DHEA treatment, the ovaries were immediately sampled from rats (n = 3 per group) under anesthesia and deep-frozen. Immunoblot analysis was performed as previously described [22]. The following primary antibodies were used for Immunoblot analysis: Rabbit anti-Bad (1:500; Cell Signaling Technology, Beverly, MA), mouse anti-ER (COX-2) (1:1,000, Santa Cruz Biotechnology), rabbit anti-iNOS (1:5,000; Cell Signaling Technology), rabbit anti-ATG (1:1,500; Enzo Life Sciences, Farmingdale, NY), mouse anti-NQO1 (1:1,500; Cell Signaling Technology), rabbit anti-p-IκBα, rabbit anti-p-IFN-κB p65 (1:1,500; Cell Signaling Technology), rabbit anti-p-IκBα, rabbit anti-p–NF-κB p65 (1:1,500; Cell Signaling Technology), rabbit anti-nuclear factor erythroid 2-related factor 2 (Nrf2; 1:1,500), Santa Cruz Biotechnology, Santa Cruz, CA), mouse antithemine oxygenase-1 (HO-1; 1:1,500; Enzo Life Sciences, Farmingdale, NY), mouse anti-NQO1 (1:1,500, Cell Signaling Technology), rabbit antihistone H3 (1:5,000; Cell Signaling Technology), rabbit anticytochrome-oxygenase-2 (COX-2) (1:1,000, Santa Cruz Biotechnology), rabbit anti-iNOS (1:1,000, Santa Cruz Biotechnology), mouse anti-ER alpha (ERs) (1:1,000, Santa Cruz Biotechnology), and mouse anti-ERα (1:500, Santa Cruz Biotechnology) were used as primary antibodies. Immunoblot images were quantified using Image J analysis software (JAVA image processing program, NIH, Bethesda, MD).

2.10. Real time-polymerase chain reaction analysis

Twenty days after the DHEA treatment, the ovaries were immediately sampled from rats (n = 6 per group) under anesthesia and deep-frozen. Real-time polymerase chain reaction (RT-PCR) was performed using a SYBR Green PCR Master Mix (Applied Biosystems, Franklin Lakes, NJ) as previously described [19]. Fold-induction was calculated using the 2ΔΔCT method as previously described [24]. Expression levels of each gene were normalized using that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primer was used for real time RT-PCR: Aromatase forward: 5’-CTC CTC CTC ATT CGG AAT TGT-3’; reverse: 5’-TCT GCC ATG GGA AAT GAG AG-3’.

2.11. RAW264.7 cell culture

RAW264.7 cells (1 × 10⁶ cells/mL) were seeded into a 96-well plate and treated with ginsenosides (1,10, and 100 μg/mL of KRGE, Rb1, Rg1, and Rg3(S)) in complete Dulbecco’s modified Eagle’s medium for 7 hours. These cells were then stimulated with lipopolysaccharide (Sigma-Aldrich, St. Louis, MO; 1 μg/mL) for another 6 hour or with estradiol (known as E2; Sigma-Aldrich; 20 nM) for 6 hour. Supernatants were collected and used for Western blot analysis. 2.12. Statistical analysis

All data are presented as means ± standard error of mean. The statistical analyses were performed using the SPSS, 21.0 package (SPSS Inc, Chicago, IL) for Windows. Two-sample comparisons were carried out using the Student t test, and multiple comparisons were made using a two-way analysis of variance with Tukey’s post hoc test. A statistical difference was identified at the 5% level unless otherwise indicated.

3. Results

3.1. Effects of KRGE on BW and estrous cycle of DHEA-induced PCOS rats

Metabolic syndrome is one of the main features of PCOS [1,3,4]. Thus, we first examined whether DHEA or KRGE could induce significant alteration in BW of rats. However, the increase was generally reduced in DHEA-treated group compared with age-matched sham rats.

3.2. Effects of KRGE on ovarian weight and morphology in DHEA-induced PCOS rats

Ovarian weight of rats can be increased or decreased because of PCOS [3]. Thus, we tested ovarian weight from each group after the end of the experiment. Ovarian weights in the DHEA group were increased by 40.5% (4.6 g) relative to that of the sham group and 249.3 ± 2.5 g in the DHEA + KRGE 75 group, and 249.3 ± 4.8 g in the DHEA + KRGE 150 group (Fig. 1B). Treatment with KRGE (150 mg/kg BW/day) alone did not affect BW compared with that of the sham (Fig. 1A and B).

Because regular estrous cycles are a key index of healthy ovarian function in women, we measured the stage of estrous cycle by a vaginal smear test (Fig. 1C). All rats of the sham and KRGE groups displayed regular cycles. The ratio of rats with regular cycle was 20% in the DHEA group. This ratio was recovered to 40% in the DHEA + KRGE group (150 mg/kg) (Fig. 1C). These findings suggest that pretreatment with KRGE exerts a favorable effect for the maintenance of a regular estrous cycle.
in the DHEA + KRGE 75 group and 3.4 ± 0.7 in the DHEA + KRGE 150 group (Fig. 1F–H). These results provide that pretreatment with KRGE can successfully disrupt the development of follicular cysts induced by DHEA.

3.3. Effects of KRGE on ovarian steroidogenic function following DHEA treatment

The formation of ovarian cysts and the presence of oligoovulation and hyperandrogenism are critical features of PCOS patients [1,3,4]. Thus, we investigated serum levels of testosterone, LH, FSH, and estradiol as markers for steroidogenic function in DHEA-induced PCOS rats (Fig. 1J and Supplementary data 1). Testosterone level was significantly enhanced in the DHEA group (by 848.5%) compared with that in the sham group, whereas its level was inhibited by 59.2% in the DHEA + KRGE group (Fig. 1J). However, LH or FSH level was not significantly affected by DHEA or KRGE (Supplementary data 1). Continuously, we examined serum levels of estradiol as additional marker for steroidogenic function in DHEA-induced PCOS rats (Fig. 1K). Estradiol level was significantly increased by 50.0% in the DHEA group compared with that in the sham group, while its level was reduced by 23.3% in the DHEA + KRGE group (Fig. 1K). In addition, mRNA expression level of aromatase, also called estrogen synthase, was significantly increased by 142.0% in the ovaries of the DHEA group compared with that in the sham group, while its level was reduced by 52.1% in the DHEA + KRGE group (Fig. 1L). These results provide evidence that positive regulation of KRGE on steroid hormones may reduce the induction of PCOS by DHEA.

3.4. Effects of KRGE on ovarian apoptosis following DHEA treatment

Apoptosis is known to be increased in ovaries from DHEA-induced PCOS rats [28]. Thus, we examined whether KRGE could reduce ovarian apoptosis at 20 days after DHEA treatment using TUNEL staining (Fig. 2). The number of TUNEL (+) cells was increased around cystic follicles in ovaries from the DHEA group, whereas it was reduced in the DHEA + KRGE group (Fig. 2A–D). In agreement with this result, protein expression levels of Bad (a proapoptotic factor), cleaved caspase-9 (an initiator), and cleaved caspase-3 (an executioner) were increased in ovaries (453.1%, 179.4%, and 216.5%, respectively) after DHEA treatment, whereas their expression levels were inhibited by KRGE pretreatment (43.9%, 34.1%, and 60.6%, respectively) (Fig. 2E and F). These results provide evidence that pretreatment with KRGE may inhibit ovarian apoptosis following DHEA treatment.

3.5. Effects of KRGE on macrophage infiltration and expression of inflammatory mediators

PCOS in humans is related to chronic inflammation [29]. The main pathological events shown in ovaries of PCOS are macrophage infiltration and macrophage-derived products [30]. Therefore, we measured the effects of KRGE on macrophage infiltration in ovarian tissues of PCOS (Fig. 3A–E). The mRNA expression of CD11b was elevated significantly by 312.2-fold in ovaries from the DHEA group compared with that of the sham group. However, the elevation in the mRNA expression of CD11b was reduced by 78.2% in ovaries from the DHEA + KRGE group (Fig. 3A). We also measured the distribution of macrophages in ovarian tissues by immunohistochemical assay using Iba-1 antibody as a marker of macrophages. In agreement with the alteration in mRNA expression of CD11b, the number of Iba-1 (+) macrophages was elevated in the theca cell layer of follicles and stromal tissue of the DHEA group (Fig. 3D). However, the number was reduced in the DHEA + KRGE group (Fig. 3E). Sequentially, we investigated the regulatory effect of KRGE on mRNA expression of chemokines (interleukin [IL]-8 and MCP-1), proinflammatory cytokines (IL-1β and IL-6), iNOS, and growth factors (EGF and TGF-β) in ovaries of each group at 20 days after DHEA treatment and/or KRGE treatment (Fig. 3F–I). The mRNA levels of IL-8, MCP-1, IL-1β, IL-6, and iNOS were elevated significantly by 64.6%, 11.5%, 38.7%, 8.9%, and 15.5-fold, respectively, in ovaries from the DHEA group compared with those in the sham group. However, the elevation in mRNA expressions was inhibited significantly by 69.5%, 73.0%, 70.0%, 67.4%, and 40.0%, respectively, in the DHEA + KRGE group (Fig. 3F–I). On the other hand, mRNA expression levels of EGF and TGF-β were reduced significantly by 60.0% and 40.0%, respectively, in the DHEA

Fig. 1. Effect of KRGE on BW gain and estrous cycle in DHEA-induced PCOS rats. (A and B) BW was measured daily during the experimental period. Growth curves for the period of treatment for sham, DHEA, DHEA + KRGE (75 and 150), and KRGE (150) groups (A) and BW at Day 20 after DHEA treatment (B). (C) Estrous cycle was determined by vaginal smear test daily following DHEA treatment. (D) Ovarian weight was measured at Day 20 after DHEA treatment. (E–I) Ovaries were performed by H&E staining at Day 20 after DHEA treatment. Sham (E), DHEA (F), DHEA + KRGE 75 (G), DHEA + KRGE 150 (H), and KRGE (I) groups. (J and K) Serum levels of testosterone (J) and estradiol (K) were measured by ELISA. (L) mRNA expression levels of aromatase in ovaries were measured by real time PCR. Values represent the mean ± SEM. *p < 0.05 and **p < 0.01 versus Sham group; #p < 0.05 and ##p < 0.01 versus DHEA group. Scale bar = 100 μm. FSH, follicle-stimulating hormone; LH, luteinizing hormone; KRGE, Korean Red Ginseng extract; DHEA, dehydroepiandrosterone; BW, body weight; H&E, hematoxylin-eosin; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; PCOS, polycystic ovarian syndrome; SEM, standard error of mean.
However, the effect of KRGE remains unclear. Thus, we examined representative in

Fig. 2. Effect of KRGE on ovarian apoptosis in DHEA-induced PCOS rats. (A-D) The level of apoptosis was measured using ovarian sections by TUNEL staining at day 20 after DHEA treatment. Sham (A), KRGE 150 (D), DHEA (B), and DHEA + KRGE 150 (C) groups. (E-F) Protein expression level of Bad, cleaved caspase-9, cleaved caspase-3 was measured in ovaries by immunoblot analysis at Day 20 after DHEA treatment (E) and quantified (F). Values represent the mean ± SEM. Scale bar = 100 μm. **p < 0.01 and ***p < 0.001 versus Sham group; *p < 0.05 and **p < 0.01 versus DHEA group. KRGE, Korean Red Ginseng extract; DHEA, dehydroepiandrosterone; PCOS, polycystic ovarian syndrome; SEM, standard error of mean; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

group compared with those in the sham group, but their levels were significantly elevated by 0.7-fold and 0.7-fold, respectively, in the KRGE group (Fig. 3K and L). KRGE itself did not exert a significant effect on the expression of these mediators (Fig. 3). These findings suggest that KRGE can inhibit macrophage infiltration and inflammatory response in the ovarian tissue of PCOS model.

3.6. Antinflammatory and antioxidant effects of KRGE in the ovarian tissue of DHEA-induced PCOS model

KRGE and ginsenosides (Rb1, Rd, Rg1, etc.) have an antinflammatory action through prevention of the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway, a representative inflammation mechanism in various diseases [11,31,32]. However, the effect of KRGE remains unclear. Thus, we examined whether KRGE could inhibit the upregulation of the NF-κB pathway in ovaries following DHEA treatment. Phosphorylation levels of IkBz and NF-κB were significantly increased by 119.5% and 713.4%, respectively, in ovaries at 20 days after the DHEA treatment compared with that of the sham group, whereas their phosphorylation levels were significantly decreased by 22.5% and 15.7%, respectively, by pretreatment with KRGE (150 mg/kg/day). KRGE treatment alone did not induce significant alteration of the NF-κB pathway (Fig. 4A–C). These results indicate that KRGE exerts antiinflammatory activity in ovaries after DHEA treatment via inhibiting the NF-κB pathway.

DHEA can increase oxidative stress in a human cell line during differentiation [33]. KRGE and ginsenosides have antioxidative effects through Nrf2 transcriptional stimulation in various diseases [23,34]. However, the antioxidant effects of KRGE on DHEA-induced PCOS in the rat model are unclear. In the present study, Nrf2 protein expression was markedly increased in ovaries of the DHEA group (43.6%) compared with that of the sham group. Its expression was further increased by pretreatment with KRGE (150 mg/kg, 57.5%) compared with that of the DHEA group (Fig. 4A and D). Protein expression levels of phase II enzymes HO-1 and NQO-1 from immunoblot analysis were markedly upregulated by 153.7% and 84.7%, respectively, in ovaries in the DHEA group. Such upregulation in protein expression were further enhanced by 28.6% and 63.3%, respectively, in the DHEA + KRGE group (Fig. 4A, E, and F). Taken together, these findings indicate that KRGE exerts antioxidant activity through upregulation of the Nrf2 pathway in ovaries of the DHEA-induced PCOS rat model.

3.7. Antinflammatory and antioxidant effects of KRGE in lipopolysaccharide-induced RAW264.7 cells

We demonstrated antinflammatory and antioxidant activities of KRGE in DHEA-induced PCOS in rats (Figs. 1–4). To further understand both activities of KRGE, we performed in vitro studies using lipopolysaccharide-induced RAW264.7 cells. KRGE inhibited the elevation of protein expression of inflammatory enzymes COX-2 and iNOS (Fig. 5A–C), the p-IkB (Fig. 5A and D), and p–NF-κB (Fig. 5A and E) after lipopolysaccharide stimulation. However, KRGE upregulated Nrf2 and HO-1 expression after lipopolysaccharide-induced RAW264.7 cell model. Protein expression levels of phase II enzymes COX-2 and iNOS (Fig. 5A and E) after lipopolysaccharide stimulation. However, KRGE upregulated Nrf2 and HO-1 expression after lipopolysaccharide stimulation (Fig. 5A, F, and G). Sequentially, to further investigate the mechanism of KRGE, we examined effects of representative components (ginsenosides) in the same in vitro study. Interestingly, ginsenoside Rb1, Rg1, and Rg3(s) prevented the elevation in protein expression of cytosolic p-IkB and nuclear p–NF-κB induced by lipopolysaccharide stimulation (Fig. 5H–J), whereas they upregulated Nrf2 nuclear translocation after lipopolysaccharide stimulation (Fig. 5H, K, and L). These results suggest that KRGE might alleviate DHEA-induced polycystic ovarian syndrome in rats via its antinflammatory and antioxidant activities.

Additionally, to examine whether antinflammatory effects of KRGE might be related to estrogen expression, we investigated the effect of KRGE on protein expression of ERα and ERβ in the lipopolysaccharide-induced RAW264.7 cell model. Protein expression levels of ERα and ERβ were increased by lipopolysaccharide stimulation, whereas their levels were reduced by KRGE (Fig. 5M–O). Protein expression levels of iNOS and p–NF-κB were increased by E2 stimulation, whereas their levels were reduced by KRGE (Fig. 5M, P, and Q). These results suggest that the antinflammatory effects of KRGE might be associated with direct or indirect effect of KRGE on estrogen expression.

4. Discussion

Because PCOS is associated to metabolic disturbances, chronic inflammation, and oxidative stress, it can affect quality-of-life and
Thus far, no therapeutic intervention has been successful in preventing or treating PCOS. KRG is one of the most important medicinal plants for patients with age-related disorders in traditional Oriental medicine. But, the effect and underlying mechanism of KRGE on the female reproductive disorders has not been analyzed systematically. Here, we demonstrated beneficial effects of KRGE in the DHEA-induced PCOS rat model via its antiinflammatory and antioxidant activities. Thus, KRGE has preventive and therapeutic potential for PCOS.

The DHEA-induced PCOS rat model mimics the main features of PCOS in women, although its detailed biology remains unknown. Our DHEA-induced PCOS rat model exhibited increased weights of animals and ovaries, corresponding to previous reports. Here, pretreatment with KRGE prevented the increase in BW and ovarian weight of rats with PCOS, corresponding to improved PCOS in rats. These possibilities were partially explained by the finding that exercise and Oriental medicine Kyung-Ok-Ko can reduce these factors associated with improved PCOS. Our results suggest that KRGE may prevent increased weights of the body and ovaries induced by DHEA treatment.

Because DHEA can induce PCOS in animal models by inducing hyperandrogenization, the level of serum testosterone is very important following DHEA treatment. In the present study, the level of serum testosterone was increased markedly after DHEA treatment. However, the increase was significantly blocked by pretreatment with KRGE. The level of serum estradiol increase is related to formation of follicular cysts, estrous cycle arrest, altered ovarian steroidogenesis, and anovulation as a result of hyperandrogenism in PCOS patients and in DHEA-induced/testosterone-induced animal models. According to recent reports, metformin and Oriental medicine Kyung-Ok-Ko can restore ovulation disorder in PCOS patients and in DHEA-induced hyperandrogenized animal models. In agreement with these reports, pretreatment with KRGE also inhibited the enhancement in serum estradiol levels and aromatase activity in ovaries from the DHEA group, mitigated the pathology of multi-cystic ovaries, and recovered estrous cycle arrest in the DHEA group of rats in the present study. These findings provide evidence that KRGE has a favorable effect in the prevention and treatment of PCOS.

DHEA promotes the apoptosis of ovarian granulosa or theca cells via phosphoinositide-3-kinase–protein kinase B/Akt, SIRT1 (member of the sirtuin family), adenosine monophosphate-activated protein kinase, or Wnt signal pathway. In the present study, the level of serum estradiol was increased markedly after DHEA treatment compared with that of the sham treatment. However, the increase was significantly blocked by pretreatment with KRGE. These possibilities were partially explained by the finding that exercise and Oriental medicine Kyung-Ok-Ko can reduce these factors associated with improved PCOS. Our results suggest that KRGE may prevent increased weights of the body and ovaries induced by DHEA treatment.

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In the present study, KRGE reduced the number of TUNEL positive cells and inhibited protein expressions of Bad (a proapoptotic factor), cleaved caspase-9 (an initiator), and cleaved caspase-3 (an executioner) in the ovary with PCOS. These results suggest that KRGE exert antiapoptotic activity in the ovary with PCOS by inhibiting Bax/caspase-9/caspase-3 pathway. Its antiapoptotic activity has been explained by abundant previous reports. Ginseng extract and its compounds have antiapoptotic activity in MPP$_{3}^+$-induced apoptosis of PC12 cells by stimulating ERs with consequent activation of extracellular-signal-regulated kinase 1/2, Akt, and inhibition of c-Jun N-terminal kinase, p38 MAPK [45]. They also have antiapoptotic activity in neuroblastoma cells via ER β-mediated phosphatidylinositol-3 kinase/Akt signaling [46].

PCOS is related to a chronic inflammatory processes [29]. Its main mechanism is macrophage contribution [30]. Ovarian macrophages can secrete proinflammatory cytokines/chemokines and
trophic factors in normal or abnormal processes of the ovary. These mediators released from macrophages can regulate tissue remodeling and apoptosis implicated in folliculogenesis, ovulation and corpus luteum/albicans formation [30]. Here, KRGE blocked infiltration of Iba-1 (+) cells (macrophages) in ovarian stroma of DHEA-induced PCOS rats, corresponding with increased mRNA expression of Iba-1 in ovaries (Fig. 3B–E). Because macrophage infiltration has a critical activity in PCOS patients [30], numerous studies have reported alteration in expression of inflammatory mediators in serum, follicular fluid, or ovarian tissue of PCOS patients. Follicular fluid and serum levels of IL-6 and TNF-α are increased in nonobese/ nonobese PCOS patients [47]. Serum levels of MCP-1 and macrophage inflammatory protein-1 alpha are increased in PCOS patients [48]. Activities of iNOS and COX-2 are increased in ovaries of PCOS patients [38,49]. Activities of iNOS and COX-2 are inhibited by metformin treatment [38]. Interestingly, here, pretreatment with KRGE significantly inhibited the stimulation of the NF-κB signal pathway and the increase in mRNA levels of IL-1β, IL-6, IL-8, MCP-1, and iNOS in ovaries from PCOS rats, whereas KRGE recovered reduced mRNA expression of EGF and TGF-β in ovaries from PCOS rats (Fig. 3F–I). In agreement with these results from in vivo study, KRGE and ginsenosides (Rb1, Rg1, and Rg3(5)) also inhibited the elevation in protein expression of inflammatory enzymes COX-2 and iNOS, cytosolic p-IκB, and nuclear p–NF–κB in lipopolysaccharide-induced RAW264.7 cell model (Fig. 5A–E). Conclusively, KRGE has an anti-inflammatory activity in ovaries of the DHEA-induced PCOS model. Therefore, targeting the inflammatory process such as activation of NF-κB signaling and/or production of inflammatory mediators using KRGE and its components might be a therapeutic alternative to current treatment.

Oxidative stress is characterized by an imbalance between production and scavenging of reactive oxygen species [50]. Oxidative stress is implicated in PCOS pathogenesis because women with PCOS display more serious oxidative stress compared with healthy woman [51]. Because transcription factor Nrf2 is responsible for strong antioxidant activity, which protects various cells from oxidative stress, targeting Nrf2 might exert a pivotal role in protection against various inflammatory disorders [52]. Given that KRGE could stimulate Nrf2 and its products (HO-1 and NQO-1) in ovaries from DHEA-induced PCOS rats (Fig. 4) and that KRGE and ginsenosides (Rb1, Rg1, and Rg3(s)) upregulated Nrf2 nuclear translocation in lipopolysaccharide-induced RAW264.7 cell model (Fig. 5), it is conceivable that triggering the Nrf2 pathway by KRGE could inhibit the NF-κB pathways, thus contributing to KRGE’s anti-inflammatory effect to protect ovaries against DHEA-induced hyperandrogenization. These findings might be directly/indirectly explained by the antioxidant effect saponin metabolite Rh3 of and ginsenoside Rg1 in cultured cells through Nrf2 stimulation [53,54] and its anti-inflammatory activity (decreased expression of cytokines and chemokines) by suppressing the NF-κB pathways through Nrf2 stimulation [34,55,56]. Although our results suggest that KRGE might suppress the activation of the NF-κB pathways via Nrf2 mechanism, reciprocal interaction between Nrf2 and NF-κB activities remains to be clarified. Nevertheless, our results suggest that KRGE can directly or indirectly exert a potent preventive and therapeutic effect in the DHEA-induced PCOS rat model by its anti-inflammatory and antioxidative effects possibly through activating the Nrf2 pathway and suppressing the NF-κB pathways.

P. ginseng, KRG, or its major active ingredients such as ginsenosides, polysaccharides, and gintonin can enhance physical strength, prevent aging, increase vigor [11–13], and induce immune, endocrine, cardiovascular, nervous, and cancer-related benefits associated with its anti-inflammatory and antioxidant activities [57]. KRGE can reduce the number of antral follicles and increase the number of corpora lutea in polycystic ovaries associated with regulation of NGF expression in an estradiol valerate-induced PCOS rat model [18]. Rg1 can enhance the antiaging capacity of ovary and fertility abilities of premature ovarian failure mice through enhancing antiinflammatory and antioxidant capacities of the ovary [58]. Ginsenosides can activate proliferation of granulosa cells from chicken prehierarchical follicles through PKC (protein kinase C) activation and upregulation of cyclin gene expression [59]. Oral administration of Rh2, a component of red ginseng, has strong inhibitory effects on human ovarian cancer cell growth in nude mice [60]. Taken together, the anti-PCOS effect of KRGE in the present study might be because of a combination action of various components. Although it remains unknown which components in KRGE exert positive effect on PCOS, the present findings have academic value as basic data for further study.

5. Conclusions

We confirmed that KRGE had beneficial effects in a DHEA-induced PCOS rat model. Pretreatment of KRGE significantly inhibited the enhancement of the weight of bodies and ovaries, size and number of follicular cysts, and serum levels of testosterone and estradiol following DHEA induction associated with its anti-inflammatory effects (reduced infiltration of macrophages and reduced mRNA expression of inflammatory mediators, enhanced growth factors, and inhibited NF-κB pathway) and antioxidant activity (Nrf2, HO-1, and NQO-1) in the ovary. Taken together, KRGE might synergistically prevent DHEA-induced PCOS via anti-inflammatory and antioxidant activities, suggesting its preventive and therapeutic potential for PCOS.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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