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STEROIDOGENIC EFFECTS OF KOREAN RICE BRAN EXTRACT ON MOUSE NORMAL LEYDIG CELLS VIA MODULATION OF STEROIDOGENESIS-RELATED ENZYMES

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ABSTRACT

Background and objective

Many plant extracts have various effects on diseases. In particular, some medicinal plants have been shown to have steroidogenic effects in men. However, the effects of Korean rice bran extract (RBE) on steroidogenesis in men have not been reported previously. Accordingly, in this study, we investigated the effects of RBE (*Oryza sativa* L.) on mouse normal Leydig cells (TM3), which produce testosterone with increasing levels of key enzymes (e.g., StAR, CYP11A1, and CYP17A1) for male steroidogenesis.

Material and methods

Using mouse normal Leydig cells (TM3), we determined whether RBE had steroidogenic effects by evaluating changes in the levels of steroidogenesis-related enzymes, such as StAR (steroidogenic acute regulatory protein), CYP11A1, and CYP17A1, using quantitative real-time PCR, western blotting analysis, and ELISA method.

Results

RBE significantly enhanced mRNA and protein levels of StAR, CYP11A1, and CYP17A1, thereby enhancing synthetic testosterone levels in mouse Leydig TM3 cell supernatants. These findings indicated that RBE increased the levels of steroidogenic enzymes to modulate steroidogenesis.

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Conclusion
RBE enhanced mRNA and protein levels of key enzymes including StAR, CYP11A1, and CYP17A1 for male steroidogenesis. These changes could induce testosterone production by TM3 cells *in vitro*. Thus, RBE may be used as a food additive or medicinal plant for the treatment of diseases characterized by insufficient testosterone (e.g., late-onset hypogonadism).

Keywords: *Korean rice bran extract; Oryza sativa L.; medicinal plant; steroidogenesis; late-onset hypogonadism*

INTRODUCTION

Testosterone is largely synthesized from Leydig cells found in testis. Firstly, cholesterol is used as substrate for steroidogenesis.[1] In aging men, the levels of blood testosterone decrease owing to the reduced ability of Leydig cells to produce testosterone.[2] The rate-limiting step during testosterone biosynthesis is the process of transferring the substrate cholesterol from the outer mitochondrial membrane into the inner mitochondrial membrane, thereby initiating the steroidogenesis.[3] In previous studies, it was reported that StAR (steroidogenic acute regulatory protein) plays critical roles during this step by facilitating mitochondrial cholesterol transfer and that StAR protein expression levels affect testosterone production in Leydig cells, strongly.[4] The substrate cholesterol is metabolized by P450scc (cytochrome P450 cholesterol side-chain cleavage enzyme) into pregnenolone which is further metabolized by CYP11A1 and HSD3B in the mitochondria and CYP17A1 and HSD17B in the smooth endoplasmic reticulum to produce testosterone.[2]

However, in aged Leydig cells, several cellular changes, including reducing luteinizing hormone-stimulated cAMP production and down-regulating StAR, the downstream steroidogenic enzyme CYP11A1 (mitochondria), and CYP17A1 (smooth endoplasmic reticulum) have been shown to collectively decrease overall testosterone synthesis through the steroidogenic pathway.[5,6]

Therefore, a clinically strategic approach would be to block deterioration of natural aging-related decreases in testosterone. However, oral administration of testosterone or direct injection of artificial testosterone may result in side effects and it is not generally considered highly sustainable as a general healthcare measure.[7] Thus, we have attempted the identification of natural plant compounds in food items and food supplements that reduce side effects at least and increase testosterone biosynthesis in Leydig cells effectively.[8]

Oryza sativa L. has been used as a food additive and has anti-oxidative and antimicrobial effects on mold and yeast.[9] Moreover, administration of rice bran extract (RBE) in mice improves sleep efficiency and sleep onset.[10] In RBE, the most abundant phenolic compounds or polyphenols are found as plant metabolites in plant tissues.[11] *In vitro* studies of plant extracts have shown that these preparations are promising because of their high antioxidant and antimicrobial activities. Rice bran phenolic extract (RBPE) also protects the liver by inactivating the endotoxin/Toll-like receptor 4/nuclear factor-κB pathway and by repressing inflammatory responses; therefore, intake of RBPE or brown rice may be an effective approach for mitigating alcoholic liver injury.[12]

Oryza sativa L. is consumed as common foodstuff by people in South Korea. We have been interested in studying the testosterone-increasing activity of this food, with a focus on the effects in TM3 mouse Leydig cells. We hypothesize that RBE may have potential applications in alleviating late-onset hypogonadism (LOH) or spermatogenesis

in aging men. However, definitive reports of the steroidogenic capacity of RBE *in vitro* have not yet been published. Accordingly, in this study, we evaluated the steroidogenic effects of RBE in TM3 (mouse Leydig) cells through analyses of mRNA, protein, and testosterone levels.

MATERIALS AND METHODS

Plant Materials

Fresh Korean RBE (*Oryza sativa* L.) was purchased from a local market in Korea. All samples were washed several times and dried in an oven at 40ºC for 3 days. The plant materials were put through a grinder to yield fine powder.

Preparation of Plant Extracts

For plant extract preparation, dry plant materials were isolated twice by adding 10-fold volume of water for 3 h by refluxing and then they were filtered (Whatman, Little Chalfont, UK). After filtration, aqueous extracts were concentrated under reduced pressure using a rotary vacuum evaporator (Eyela CCA-1111; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and lyophilized (Bondiro; IlShin BioBase, Seoul, Korea) for obtaining powder.

TM3 Cell Culture

TM3 (mouse Leydig) cells were purchased from ATCC (American Type Culture Collection; ATCC No. CRL–1714; ATCC, Manassas, VA, USA) and they were cultured in DMEM/F12 medium containing 2.5 mM L-glutamine, 0.5 mM sodium pyruvate, 1.2 g/L sodium bicarbonate, 15 mM HEPES (92.5%), 5% horse serum, and 2.5% fetal bovine serum, supplemented with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C cell incubator. Every 2 days, the new medium was supplied.

TM3 Cell Viability Assay

In order to measure the effects of RBE on TM3 cell viability, we used an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (Cell Counting Kit-8; cat. no. CK04; Dojindo Molecular Technologies, Inc., Rockville, IL, USA), according as the manufacturer's protocols. All experiments were performed in triplicate.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

TM3 cells were seeded in six-well plates (5.0×10^5 cells/well) and cultured for 24 h before treatment. The fresh medium was changed and they were treated with or without RBE (0, 1, 10, 25, or 50 μg/mL). Total RNA was isolated from the cell pellets using an RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions, cDNA was made by a high-capacity cDNA reverse transcription kit (QuantiTect Reverse Transcription kit; Qiagen). Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was performed with a Rotor-Gene SYBR Green PCR kit (Qiagen) on a Rotor-Gene Q PCR machine (Qiagen) with a two-step cycling protocol, as follows: the denaturation step, at 95°C for 5 s and annealing/extension step at 60°C for 10 s (40 cycles). Cycle threshold (Ct) values were within 0.1 among triplicates. Primers for qRT-PCR were designed to target StAR, CYP11A1, CYP17A1, and 18s rRNA and the results were quantified by the $2^{-\Delta\Delta Ct}$ method after normalization to 18s rRNA expression as an internal control. The primer sequences were as follows:

StAR-forward
(5′-TCTCTAGTGTCTCCCACTGCATAGC-3′),
StAR-reverse
(5′-TTAGCATCCCCTGTTCGAGCT-3′),
CYP11A1-forward
(5′-ACATGGCCAAGATGGTACAGTTG-3′),
CYP11A1-reverse
(5′-ACGAAGCACCAGGTCATTCAC-3′),
CYP17A1-forward
(5′-CTCCAGCCTGACAGACATTCTG-3′),
CYP17A1-reverse
(5′-TCTCCCACCGTGACAAGGAT-3′),

18s rRNA-forward
(5′-GAGGCCCTGTAATTGGAATGAG-3′), and
18s rRNA-reverse
(5′-GCAGCAACTTTAATATACGCTATTGG-3′).

The experiments were done in triplicate.

Western Blot Assay

TM3 cell were reacted with cell lysis buffer (Cell Signaling Technology, Danvers MA, USA #9803). Protein amount is normalized with BCA protein assay kit (Thermo Scientific, Rockford, IL. USA, #23225). Next, 20 μg cell lysate was separated by 10% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and electro-transferred onto NC (nitrocellulose) membranes, as described elsewhere. The blotted membranes were then washed in TBST(Tris-buffered saline: 20 mM Tris-HCl, pH 7.6, containing 137 mM NaCl and 0.05% [v/v] Tween 20), blocked with 5% skimmed milk for 1 h at room temperature with shaking, reacted with primary antibodies (1:1000) at 4°C for 16 h with shaking. The primary antibodies were used with anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-47778), anti-StAR (Abcam, USA; ab203193), anti-CYP11A1 (Abcam, USA; ab175408), and anti-CYP17A1 (Abcam, USA; ab115022). Subsequently, the membranes were rinsed with TBST buffer several times (10 min each) and then reacted with secondary antibodies (1:5000) labeled with HRP at room temperature for 1 h with shaking. The membranes were washed with TBST buffer several times (10 min each) and the signals were detected by an ECL solution (enhanced chemiluminescence western blot kit: EzWestLumi plus; ATTO Corporation, Motoasakusa, Tokyo, Japan) and quantified using an ImageQuant LAS 4000 mini machine (GE Healthcare).

Enzyme-Linked Immunosorbent Assay Method for Detecting Testosterone

In order to detect synthetic testosterone contents in TM3 cell supernatants, at first TM3 cells were seeded in cell dishes and cultured for 24 h before experiment. The medium was discarded and the cells were treated in RBE (0, 1, 10, 25, and 50 μg/mL) for another 48 h. TM3 cell supernatants were frozen before use in experiments or the fresh cell supernatants were used directly to Enzyme-Linked Immunosorbent Assay method for detecting testosterone (ELISA). ELISA was performed using a Parameter Testosterone Assay kit (KGE010; R&D Systems, Inc.) according to the manufacturer's instructions. Detection was carried out by measuring the absorbance at 450 nm with microplate reader. All experiments were conducted in triplicate.

Statistical Analysis

All experiments were repeated three times and all data are presented as means ± standard deviations. To identify significant differences (SD) between groups, we used one-way analysis of variance followed by the Fisher's least significant difference (LSD) multiple comparison test in GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Results with P values of less than 0.05 (*, $p<0.05$) were considered statistically significant.

RESULTS

RBE Did Not Affect Cell Viability in TM3 Cells

To examine whether RBE affected the viability of TM3 cells, they were incubated with varying concentrations of RBE (0, 1, 5, 10, 25, or 50 μg/mL) for 24 h (Figure 1A) and 48 h (Figure 1B). MTT assays resulted that RBE did not significantly affect cell viability (Figure 1).

RBE Induced StAR, CYP11A1, and CYP17A1 mRNA Expression of Steroidogenesis-Related Genes in TM3 Cells

Next, RBE was treated with TM3 cells at different concentrations for 24 h and changes were assessed in mRNA levels by qRT-PCR. The steroidogenesis-related genes *StAR*, *CYP11A1*, and *CYP17A1* were upregulated by 18.46, 31.2, and 30.8 times, respectively, following treatment with

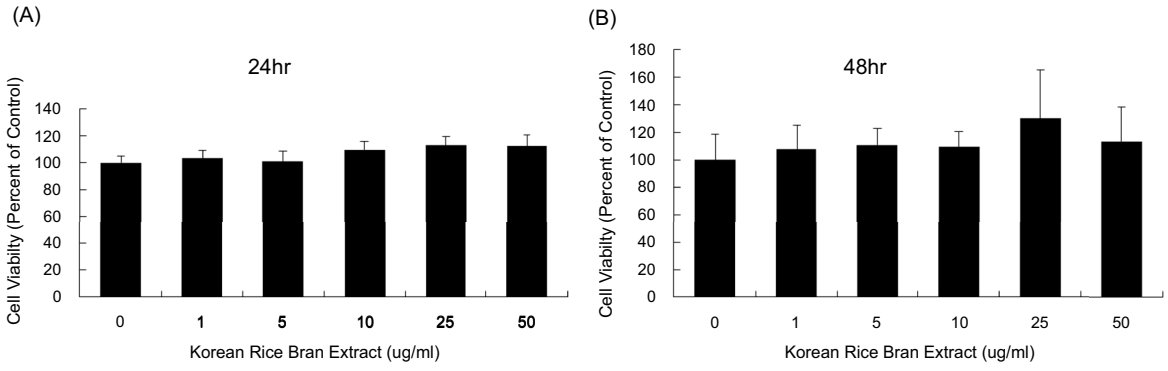

FIGURE 1 Viability of TM3 (mouse Leydig) cells treated with RBE. TM3 Cells were treated with 0, 1, 5, 10, 25, and 50 μg/mL of RBE for 24 h (A) and 48 h (B). All experiments were conducted in triplicate.

1 μg/mL RBE (Figure 2A–2C), compared with that in the control group. Thus, these findings indicated that RBE activated steroidogenic enzymes at specific concentrations *in vitro* and highlighted the role of RBE in male steroidogenesis in mouse Leydig cells.

RBE Increased StAR, CYP11A1, and CYP17A1 Protein Expression of Steroidogenesis-Related Genes in TM3 Cells

TM3 cells were then treated with 0, 1, 10, 25, or 50 μg/mL RBE for 48 h and protein expression was assessed by western blotting. Similar to the qRT-PCR data, we found that StAR, CYP11A1, and CYP17A1 protein levels were increased following treatment with RBE (Figure 3A–3D). Thus, these findings confirmed that RBE activated the steroidogenic enzymes STAR, CYP11A1, and CYP17A1 *in vitro* and further supported that RBE affected male steroidogenesis in mouse Leydig cells.

RBE Enhanced Testosterone Synthesis in TM3 Cells

Next, we evaluated changes in the levels of testosterone produced following treatment with RBE for 48 h in mouse Leydig cells. Testosterone levels were significantly increased following treatment with 1 μg/mL and 25 μg/mL RBE (Figure 4). These findings suggested that RBE enhanced testosterone levels directly involved in testosterone synthesis *in vitro*.

DISCUSSION

Rice (*Oryza sativa* L.) is a staple food in Asian countries, including South Korea. Rice bran, as the most abundant and valuable by-product of the rice milling process, is a unique source of bioactive phytochemicals with potential applications in various fields. Indeed, RBE possesses large amounts of phytochemicals which can be extracted and used in foods, cosmetics, and pharmaceuticals.[13] These include liposoluble compounds (such as γ-oryzanol, tocopherols, tocotrienols, and phytosterols) and phenolic compounds. Many secondary metabolites are phenolic compounds that are widely distributed in plant tissues[14] and have been shown to have multiple bioactivities, such as anti-oxidative, antimicrobial,[9,15] antiviral,[16] and anti-inflammatory properties.[12,17]

However, the mechanisms mediating the steroidogenic effects of RBE in TM3 (mouse Leydig) cells have not been thoroughly assessed. In this study, we found that RBE increased testosterone

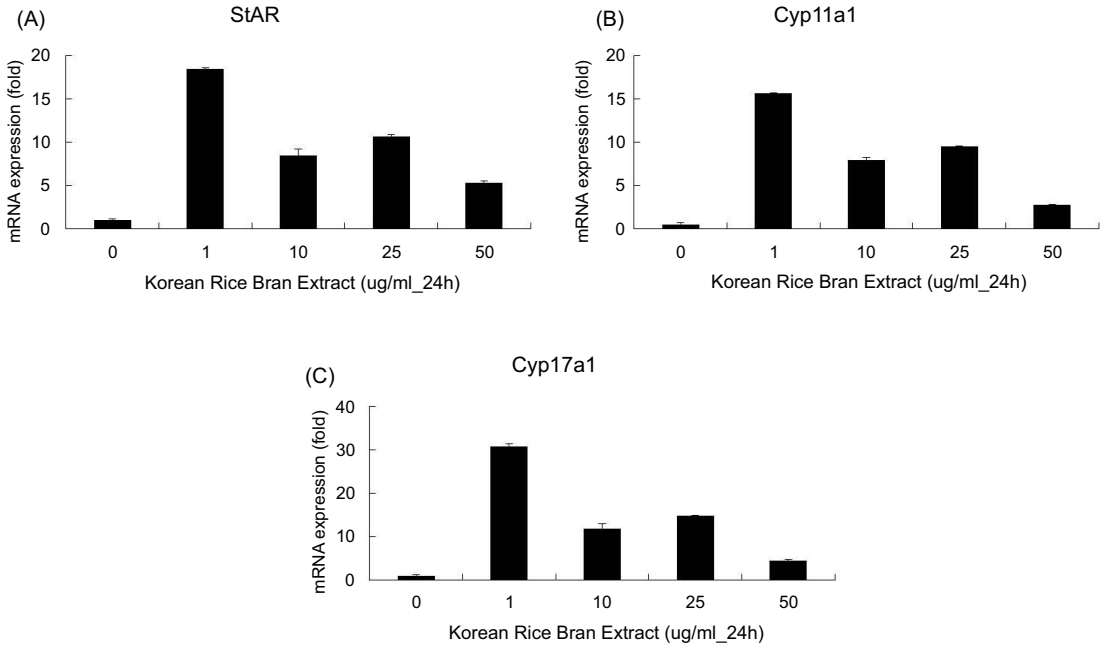

FIGURE 2 mRNA levels of *StAR*, *Cyp11a1*, and *Cyp17a1* in TM3 (mouse Leydig) cells following treatment with RBE. TM3 cells were reacted with 0, 1, 10, 25, or 50 μg/mL RBE for 24 h, and qRT-PCR was conducted with steroidogenic genes-specific primers to detect *StAR* (A), *Cyp11a1* (B), and *Cyp17a1* (C). Three mRNA levels were normalized to the expression of the 18s rRNA gene. The experiments were done in triplicate.

biosynthesis directly in Leydig cells *in vitro*. Furthermore, RBE treatment effectively increased StAR, CYP11A1, CYP17A1, mRNA, and protein levels in mouse Leydig cells and enhanced testosterone production.

Steroidogenesis in mouse Leydig cells has been reported to involve several enzymes.[2] Luteinizing hormone (LH) and the LH receptor together form the Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR) complex in response to LH stimulation. Then, the LHCGR complex stimulates cAMP for using cholesterol in the cytosol as a substrate for steroidogenesis, resulting in induction of StAR, a cholesterol transporter.[2,5,18–20] In our study, it was confirmed that *StAR* mRNA expression was increased in a concentration-dependent manner at 24 h after RBE treatment, whereas StAR protein was increased at 48 h after treatment with RBE in mouse Leydig cells. Thus, StAR expression was activated by BRE *in vitro*. StAR forms a complex with cholesterol and the outer mitochondrial membrane cholesterol-binding translocator protein in the inner membrane of mitochondria in Leydig cells regulates downstream steroidogenic enzymes (HSD3B and CYP11A1) in the mitochondria and HSD17B, HSD3B, and CYP17A1 enzymes in smooth endoplasmic reticulum (SER). It is showed that StAR, CYP11A1, and CYP17A1 expression levels were increased at the mRNA and protein levels following treatment with RBE in mouse Leydig cells. Accordingly, these findings suggested that RBE enhanced CYP11A1 and CYP17A1 expression in the mitochondria and smooth endoplasmic reticulum. Finally, we also found that RBE treatment for 48 h

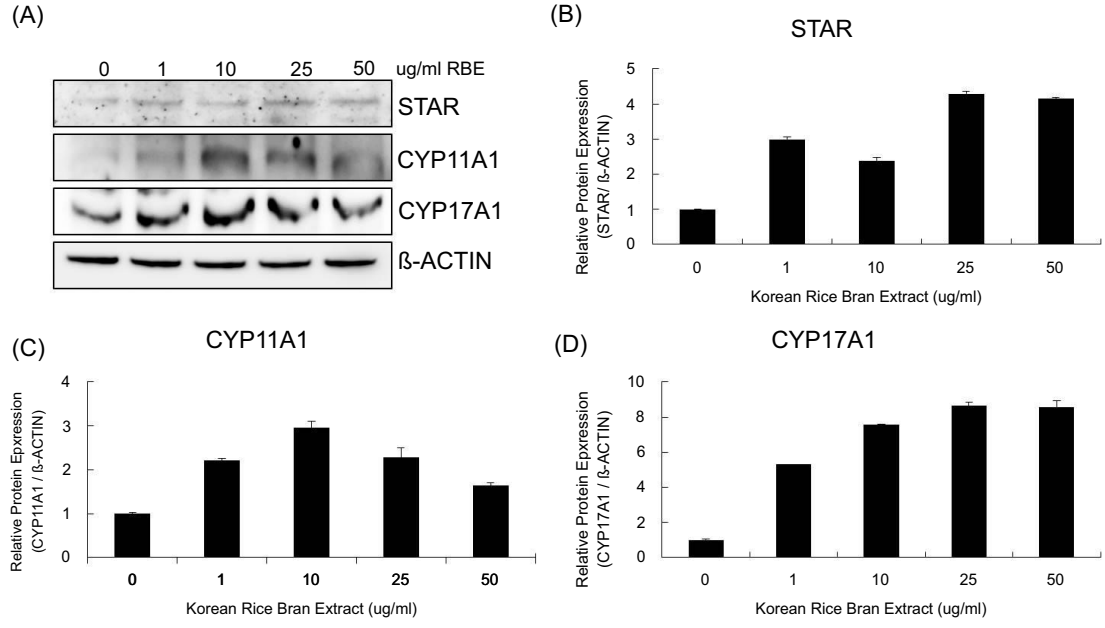

FIGURE 3 Protein levels of STAR, CYP11A1, and CYP17A1 in TM3 (mouse Leydig) cells following treatment with RBE. TM3 cells were incubated with 0, 1, 10, 25, or 50 μg/mL RBE for 48 h and western blotting analysis was performed for detection of StAR, CYP11A1, and CYP17A1 (A). Total protein was normalized with β-actin as internal control and the data were quantified (B–D). The experiments were performed in triplicate.

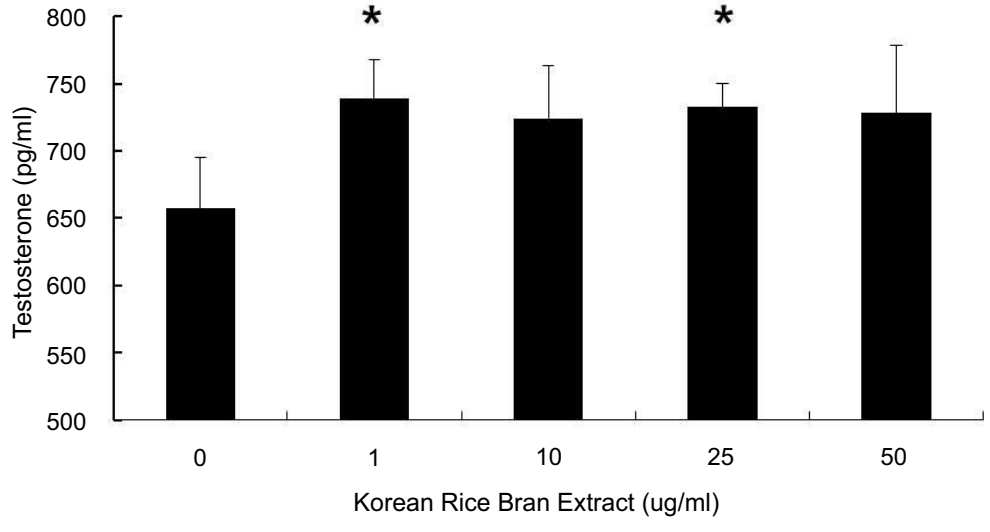

FIGURE 4 Testosterone levels in TM3 cell supernatants. TM3 cells were treated with 0, 1, 10, 25, or 50 μg/mL RBE for 48 h. TM3 cell supernatants were obtained and subjected to ELISA method. *$p < 0.05$. The experiments were performed three times.

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increased testosterone levels. Thus, our results confirmed the steroidogenic effects of RBE in mouse Leydig cells.

The pharmacological effects of many plant extracts have been reported; however, this is the first study supporting the potential steroidogenic effects of RBE as a medicinal plant in aging males. However, the bioactivities of many plant extracts have not been defined well, and information about toxicity and adverse effects is insufficient. Thus, it will be necessary to evaluate the safety, efficacy, and optimal dose of this medicinal plant prior to its applications in the clinical setting. Moreover, it will be important to perform studies of QC (quality control), standardization, and validation through various methods.

In this study, it was found that RBE treatment increased the expression of steroidogenesis-related enzymes and promoted testosterone production in Leydig cells. Thus, our findings suggested that RBE may be an alternative medicinal plant extract for improvement of diseases characterized by insufficient testosterone (i.e., male infertility, LOH and male hypogonadism). Future studies are needed to improve its clinical efficacy.

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CONCLUSION

In summary, we found that RBE treatment enhanced the mRNA and protein levels of steroidogenesis-related enzymes (i.e., StAR, CYP11A1, and CYP17A1), which facilitate the production of testosterone in TM3 mouse Leydig cells. Therefore, RBE might be used as a food additive or medicinal plant for the treatment of diseases characterized by insufficient testosterone. In further studies, the clinical efficacy of RBE will need to be investigated.

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