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Korean Ginseng Berry Extract Enhances the Male Steroidogenesis Enzymes *In Vitro* and *In Vivo*

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Purpose: Testosterone hormonal replacement is the most commonly prescribed solution for men with reproductive issues; however, this treatment has various drawbacks. Hence, the identification of a natural product that promotes steroidogenesis is urgently needed. Ginseng is a popular traditional medicine. This study aimed to investigate steroidogenic effects of Korean ginseng berry extract (GBE; Panax ginseng C.A. Meyer) *in vitro* and *in vivo*.

Materials and Methods: *In vitro* model, mouse Leydig cells were treated with varying concentrations of GBE, and the levels of steroidogenesis-related genes and proteins and testosterone were measured using western blotting, qRT-PCR, and enzyme-linked immunosorbent assay (ELISA). Similarly, in an *in vivo* model using lipopolysaccharide-injected C57BL/6J mice, expression of steroidogenesis-related genes and proteins and testosterone levels were analyzed. Additionally, sleep deprivation was used to simulate common life stressors related to late-onset hypogonadism (LOH) and the natural effects of aging. Mice were fed sham or GBE before being subjected to paradoxical sleep deprivation.

Results: *In vitro*, GBE induced steroidogenic effects by increasing the levels of enzymes associated with steroidogenesis, steroidogenic acute regulatory protein (STAR), CYP11A1, and CYP17A1. *In vivo*, GBE significantly increased mRNA and protein levels of steroidogenic enzymes. Furthermore, the synthetic testosterone levels in mouse Leydig cell supernatants and blood sera were increased. In the sleep deprivation study, mice fed GBE showed increased testosterone production and survival under such stressful conditions.

Conclusions: GBE increased mRNA and protein levels of steroidogenesis-related enzymes STAR, CYP11A1, and CYP17A1. These key enzymes induced the increased production of testosterone both *in vivo* and *in vitro*. Thus, GBE might be a promising therapeutic or additive nutritional agent for improving men's health by increasing steroidogenesis or improving LOH.

Keywords: Korean ginseng berry extract; Late-onset hypogonadism; Leydig cell; Medicinal plant; *Panax ginseng*. CA. Meyer; Steroidogenesis

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INTRODUCTION

Ginseng is one of the most popular ingredients in the library of eastern traditional medicines, and has gained much popularity in contemporary medicine as a phytochemical source to assist in various maladies, including diabetes, vascular disease, menopausal symptoms, and erectile function [1-6]. Various active components, including saponins, polysaccharides, phenols, gomisin, acidic peptides, and other various carbohydrates, have been identified in ginseng [7,8]. The principal component of interest are the ginsenosides, the concentration of which varies depending on the part of the plant, leave, stem, or berry, and the extraction method [1,9-11].

Although the ginseng root has been the primary focus in traditional oriental medicine, recent studies have highlighted the properties of the berry. Recent studies have shown the ginseng berries have comparable and effective compositions of ginsenosides [7,11-13]. Various mechanisms of action have been utilized to explain the wide spectrum of beneficial effects presented by the ginseng berries. Various studies have highlighted the antidiabetic and vascular effects of ginseng extracts; however, no definite remedial study has been presented describing its effect on male hypogonadism [1,7,14].

Late-onset hypogonadism (LOH) is a significant deterrent of quality of life in old age. It is widespread and exacerbated by common comorbidities in old age [15]. While several pathophysiologic changes affect the decrease of testosterone throughout various scenarios, including, and sometimes overlapping, vascular, hormonal, genetic and epigenetic changes, ultimately, deterioration of biochemical testosterone production is affected [16-18]. As ginsenoside compounds are capable of affecting steroidogenesis pathway from cholesterol, it is possible to rescue deteriorated steroidegenic mechanisms in LOH. Previously we have investigated similar compounds with *Taraxacum officinale* extract (TOE) [19] and Korean rice bran extract (*Oryza sativa L.*) [20] by increasing steroidogenesis-related enzymes (*i.e.*, STAR, CYP11A1, and CYP17A1), which facilitate the production of testosterone in TM3 mouse Leydig cells.

In this study, *Panax ginseng* Meyer berry water was extracted to minimize loss or altercation of major ginsenosides and was applied to Leydig cells to evaluate its effects on spermatogenesis-related enzymes *in vitro* and *in vivo*. The extract was also used to assess *in vivo* function in a fatigue-based aging model.

MATERIALS AND METHODS

1. Preparation of ginseng berry extract

Panax ginseng berry (*Panax ginseng* C.A. Meyer) was washed with water and the seeds were removed. The remaining pulp and rinds were collected, and 100% water extraction was performed under reflux conditions for 2 to 5 hours. The extract was then filtered and evaporated. The extract solution was spray dried to acquire a powdered ginseng berry extract (GBE) and was stored at -20℃ until further use. The standardized ginseng berry powder contained 5% ginsenoside Re.

2. UHPLC-LTQ-Orbitrap-MS/MS analysis

To standardize and compare ginsenoside composition, we analyzed the product of the 100% water extraction. The extract was then resuspended in 100% methanol at 2,500 ppm, and the suspensions were then analyzed by UHPLC-LTQ-Orbitrap-MS/MS analysis to detect secondary metabolites. The UHPLC system was equipped with a Vanquish binary pump H system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an autosampler and a column compartment. Phenomenex KINETEX C18 Column (Phenomenex, Torrance, CA, USA) was used perform chromatographic separation; the injection volume was 5 L. The column temperature was set to 40℃, and the flow rate was 0.3 mL/min. The mobile phase consisted of 0.1% v/v formic acid in water (A) and acetonitrile (B). The total run time was 14 minutes. Briefly, 5% of solvent B was maintained initially for 1 minute, followed by a linear increase to 100% of solvent B for over 9 minutes, and then sustained at 100% of solvent B for 1 minute, with a gradual decrease to 5% over 3 minutes. The ion-trap analysis was performed in full scan ion modes within a range of 100 to 1,500 m/z under negative- and positive-ion modes.

3. *In vitro* experiments with mouse Leydig cell (TM3) culture

Cell culture, TM3, mouse Leydig cells are purchased (ATCC No CRL – 1714; Manassas, VA, USA). Cell viability assay, to assess the effects of GBE on TM3 Leydig cell viability, MTT assay kit (cell counting kit-8, CK04) was purchased from Dojindo Molecular Technologies, INC. (Rockville, MD, USA). Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), total RNA was isolated from the cell pellets using RNeasy mini kit (Qiagen, Hilden, Germany), 1 to

3 μg of RNA were used for making cDNA, which was made by cDNA reverse transcription kit (QuantiTect Reverse Transcription kit; Qiagen). 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 seconds and annealing/extension step at 60°C for 10 seconds (40 cycles). Cycle threshold (Ct) values were within 0.1 among triplicates. The primers were designed for CYP11A1, CYP17A1, STAR, and 18s rRNA (Table 1), which yielded a $2^{-\Delta\Delta Ct}$ value and used for normalization. The experiments were performed in triplicate. For western blotting analysis, protein amount is normalized with BCA protein assay kit (#23225; Thermo Scientific, Rockford, IL, USA). Next, 20 μg cell lysate was separated by 10% SDS-PAGE and electrotransferred onto NC membranes. The primary antibodies were used with anti-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-STAR (ab203193; Abcam; Cambridge, MA, USA), anti-CYP11A1 (ab175408; Abcam), and anti-CYP17A1 (ab115022; Abcam). Enzyme-linked immunosorbent assay (ELISA) for detecting testosterone, TM3 cell supernatants were freezing before the experiment. ELISA procedure were done according to the manufacturer's instrument (Parameter™ Testosterone Assay, # KGE010; R&D Systems®, Inc, Minneapolis, MN, USA) [19,20].

4. *In vivo* experiments with C57BL/6J mice

1) Animals

Male C57BL/6J mice (10-week-old on average) were used. All mice were housed in a controlled environment (25°C±1°C and 55%±5% humidity) with a 12 hours day-light cycle and access to food and water *ad libitum*. The mice were subjected to an adaptation period for one to two weeks. All experimental animal procedures were carried out in accordance of the approved animal guidelines of the Animal Care Committee at Chung-Ang University (approval number: 201900109). All efforts were made to minimize the suffering and number of animals involved. Each experimental group had eight mice which were fed 0, 50 or 100 mg/kg GBE for five days (120 h). Then, lipopolysaccharide (LPS; 1 mg/kg) was intraperitoneally injected 6 hours before sacrifice.

2) Isolation of testes and blood serum for qRT-PCR, western blotting, and ELISA

After treatment with GBE and LPS, the mice were sacrificed. For euthanasia, a container is usually prefilled with a minimum of 98% (volume) of N_2 or O_2, to induce death. After sacrifice, the whole blood was collected from the right atrium or the abdominal vena cava, allow the blood to clot at RT, removed the clot by centrifugation at 1,000 to 2000×*g* for 10 minutes at 4°C, discarded the serum into the new tubes and stored serum at -70°C deep freezer. The collected serum was not freeze-thaw cycles before experiments. After collecting blood, the testis were dissected, dipped them in nitrogen gas and stored them at -70°C deep freezer immediately [19,20].

3) Sleep deprivation

Alternatively, a subgroup of mice from each arm was subjected to sleep deprivation protocol. Mice were placed onto a narrow cylindrical platform, 3.2 cm in diameter surrounded by water to accomplish a partial sleep deprivation for 2 days. We provided food and water ad libitum throughout the whole experiment. This method is reported to produce selective paradoxical sleep deprivation [21].

Table 1. Primer information for qRT-PCR

Name of primers	Primer sequence (5′-3′)	PCR product size (bp)	Gene access number
m-18s rRNA Forward	5'-GAGGCCCTGTAATTGGAATGAG-3'	120bp	NR_003278.3
m-18s rRNA Reverse	5'-GCAGCAACTTTAATATACGCTATTGG-3'		
m-StAR Forward	5'-TCTCTAGTGTCTCCCACTGCATAGC-3'	121bp	NM_011485
m-StAR Reverse	5'-TTAGCATCCCCTGTTCGTAGCT-3'		
m-Cyp11a1 Forward	5'-ACATGGCCAAGATGGTACAGTTG-3'	119bp	NM_019779
m-Cyp11a1 Reverse	5'-ACGAAGCACCAGGTCATTCAC-3'		
m-Cyp17a1 Forward	5'-CTCCAGCCTGACAGACATTCTG-3'	117bp	NM_007809
m-Cyp17a1 Reverse	5'-TCTCCCACCGTGACAAGGAT-3'		

qRT-PCR: quantitative real-time reverse transcriptase-polymerase chain reaction.

5. Statistical analysis

All data are presented as the mean±standard deviation. Significant differences between groups were determined using a one-way analysis of variance (ANOVA) followed by the Fisher's least significant difference for multiple comparison, using the GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Statistical significance was considered at *p<0.05, **p<0.01 and ***p<0.001.

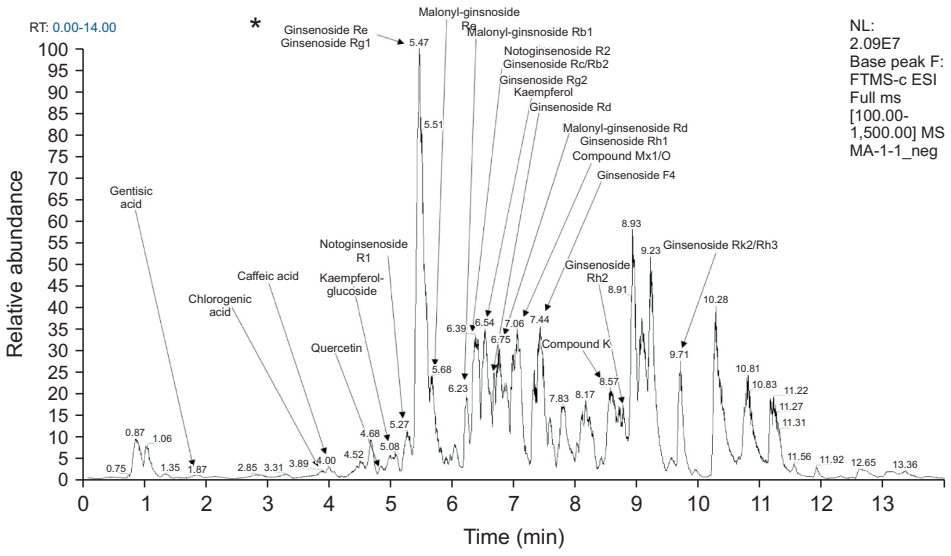

Fig. 1. Chromatogram for ginseng berry extract (100% water extract) analyzed by UHPLC-LTQ-Orbitrap-MS/MS. Ginsenoside Re (denoted by the * sign) is the most abundant ginsenoside of ginseng berry.

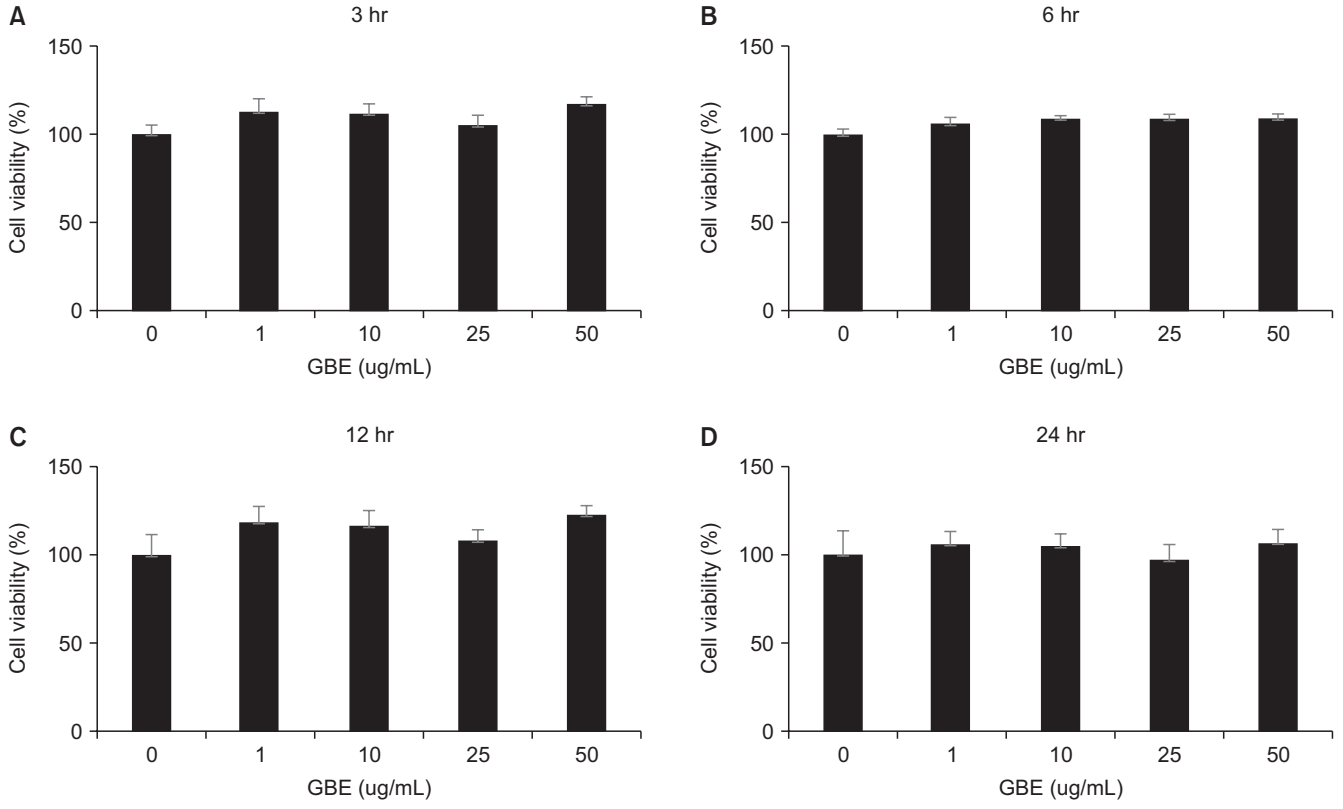

Fig. 2. Leydig cell (TM3) viability following treatment with ginseng berry extract (GBE). TM3 cells were treated with 0, 1, 10, 25, and 50 μg/mL of GBE for 3 hours (A), 6 hours (B), 12 hours, (C) and 24 hours (D). All measurements were performed in triplicate.

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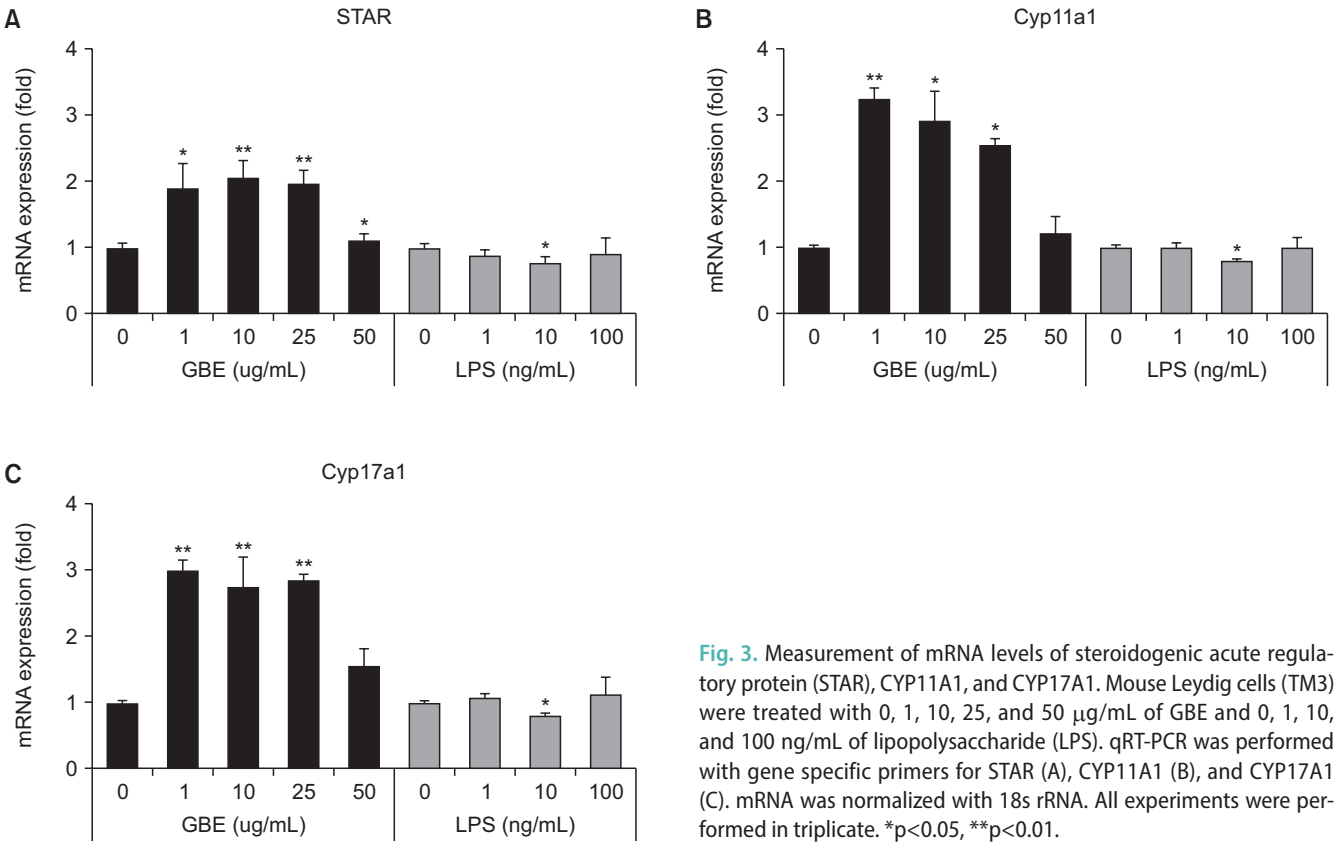

Fig. 3. Measurement of mRNA levels of steroidogenic acute regulatory protein (STAR), CYP11A1, and CYP17A1. Mouse Leydig cells (TM3) were treated with 0, 1, 10, 25, and 50 μg/mL of GBE and 0, 1, 10, and 100 ng/mL of lipopolysaccharide (LPS). qRT-PCR was performed with gene specific primers for STAR (A), CYP11A1 (B), and CYP17A1 (C). mRNA was normalized with 18s rRNA. All experiments were performed in triplicate. *p<0.05, **p<0.01.

RESULTS

1. Chromatogram of GBE

GBE was prepared using the pulp and rinds from fresh *P. ginseng* berries. The compounds of *P. ginseng* berry based on the retention time and chromatogram pattern are shown in Fig. 1. Ginsenoside Re was the most abundant ginsenoside in the ginseng berry.

2. Effect of GBE on the viability of mouse Leydig cells

To examine whether GBE affects cell viability, we treated the mouse TM3 cells with varying concentrations of GBE, 0, 1, 10, 25, or 50 μg/mL, for 3, 6, 12 and 24 hours. The MTT assay showed that GBE did not have any significant effect on cell viability (Fig. 2).

3. GBE induces mRNA, protein expression of steroidogenesis-related genes and enhances testosterone synthesis in mouse Leydig cells

We treated mouse TM3 cells, supplemented with working media, with 0, 1, 10, 25, or 50 μg/mL GBE for 24 hours, harvested the total RNA, and performed qRT-pCR. The levels of STAR, CYP11A1, and CYP17A1 were detected and expression of these three genes were increased by GBE significantly. In contrast, the mRNA levels of STAR, CYP11A1, and CYP17A1 in the LPS groups decreased in a dose-dependent manner (Fig. 3). To harvest the cellular protein, and perform western blotting analysis. The protein levels of the steroidogenic genes, STAR, CYP11A1, and CYP17A1, were detected and their expression increased on treatment with GBE (Fig. 4A). The protein levels of STAR, CYP11A1, and CYP17A1 decreased in a dose-dependent manner in the LPS treated group (Fig. 4C). The results of the western blotting analysis was quantitatively analyzed using the Image J program (National Institutes of Health, Rockvile, MD, USA) (Fig. 4B, 4D). After investigating the mRNA and protein levels of steroidogenic genes, STAR, CYP11A1, and CYP17A1, we detected the amount of synthetic testosterone in the cell supernatant following treatment with GBE for 48 hours using ELISA. The testosterone levels increased significantly on treatment with 10 μg/mL ($p<0.05$) and 25 μg/mL ($p<0.01$) GBE. In the LPS-treated group, the testosterone levels decreased significantly at 0.5, 1, 5, 10, and 50 ng/mL in a dose-dependent manner compared to the control group ($p<0.01$)

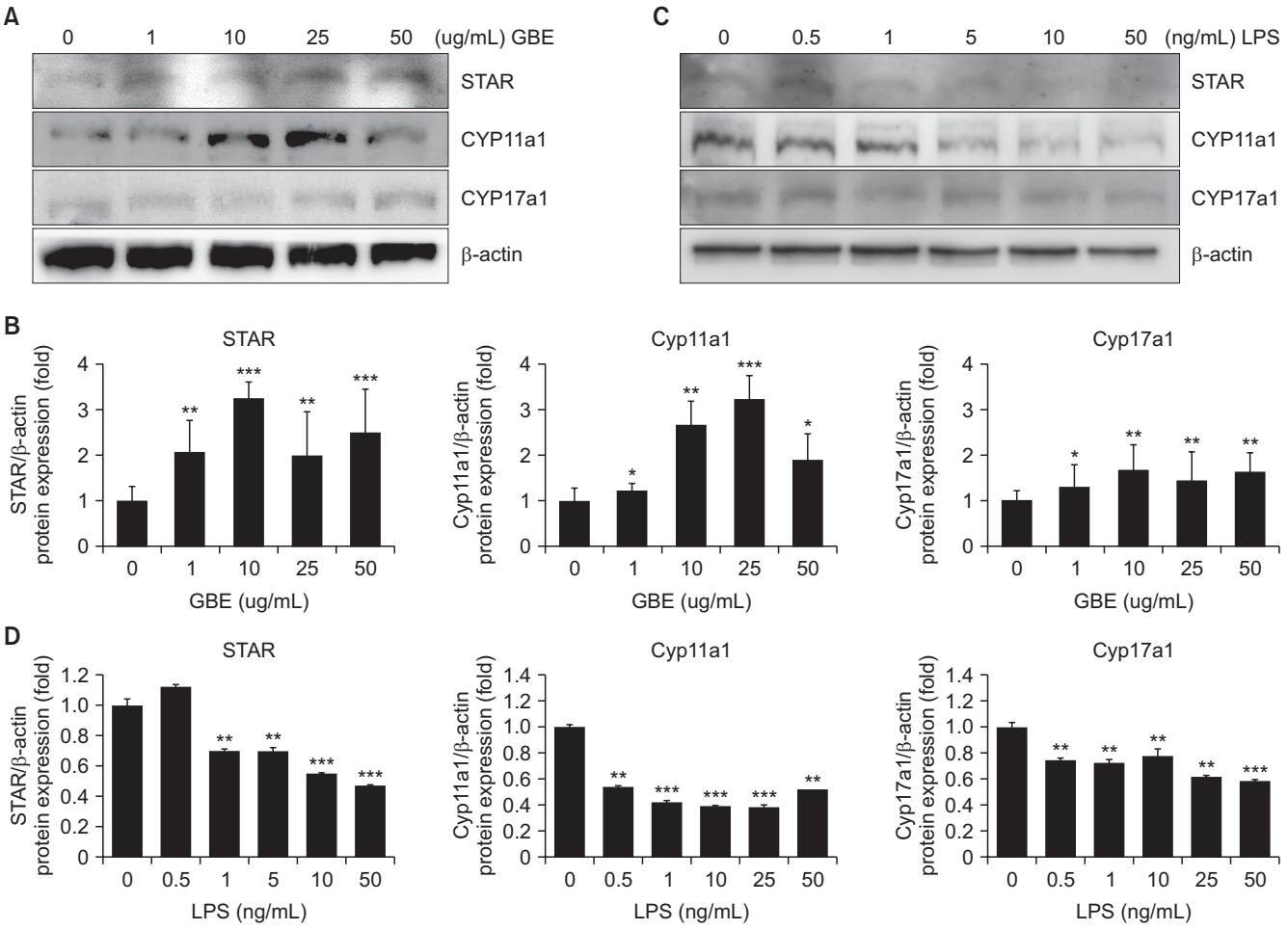

Fig. 4. Measurement of protein levels of steroidogenic acute regulatory protein (STAR), CYP11A1, and CYP17A1. TM3 cells were treated with 0, 1, 10, 25, and 50 μg/mL of ginseng berry extract (GBE) (A) and 0, 0.5, 1, 5, 10, and 50 ng/mL of lipopolysaccharide (LPS) (C). Western blotting was performed with the gene specific antibodies for STAR, CYP11A1, CYP17A1, and β-ACTIN. Total protein was normalized with the expression of the β-ACTIN gene (B and D). *p<0.05, **p<0.01, ***p<0.001.

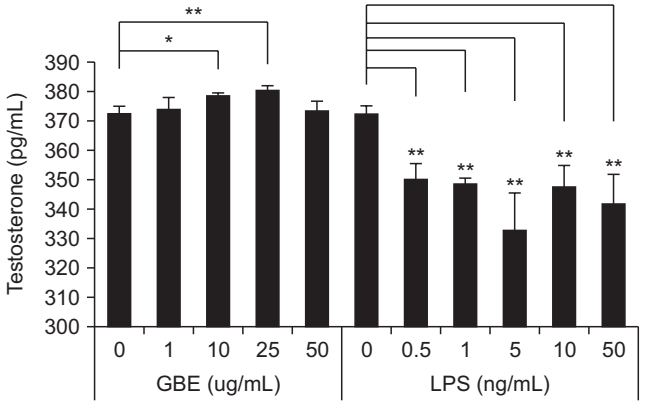

Fig. 5. Measurement of testosterone levels in TM3 cell supernatants. Mouse Leydig cells were treated with 0, 1, 10, 25, and 50 μg/mL of ginseng berry extract (GBE) and 0, 0.5, 1, 5, 10, and 50 ng/mL of lipopolysaccharide (LPS). TM3 cell supernatant was collected and used for ELISA. *p<0.05, **p<0.01.

(Fig. 5). This demonstrates that GBE is involved in male steroidogenesis in mouse Leydig cells.

4. GBE recovered mRNA, protein expression of steroidogenesis-related genes, testosterone synthesis from LPS treated group in mouse Leydig cells

Further, to determine steroidogenic effect of GBE, TM3 cells were treated with GBE in combination with LPS (5 ng/mL). The LPS-induced decrease in STAR, CYP11A1, and CYP17A1 mRNA, protein levels recovered after GBE treatment (Fig 6, 7). The LPS-induced decrease in the synthetic testosterone levels were significantly recovered by GBE ($p<0.01$) (Fig. 8). This result shows that GBE also enhanced the testosterone level directly and that GBE is involved in the synthesis of testosterone *in vitro*. Thus, GBE plays a role in male

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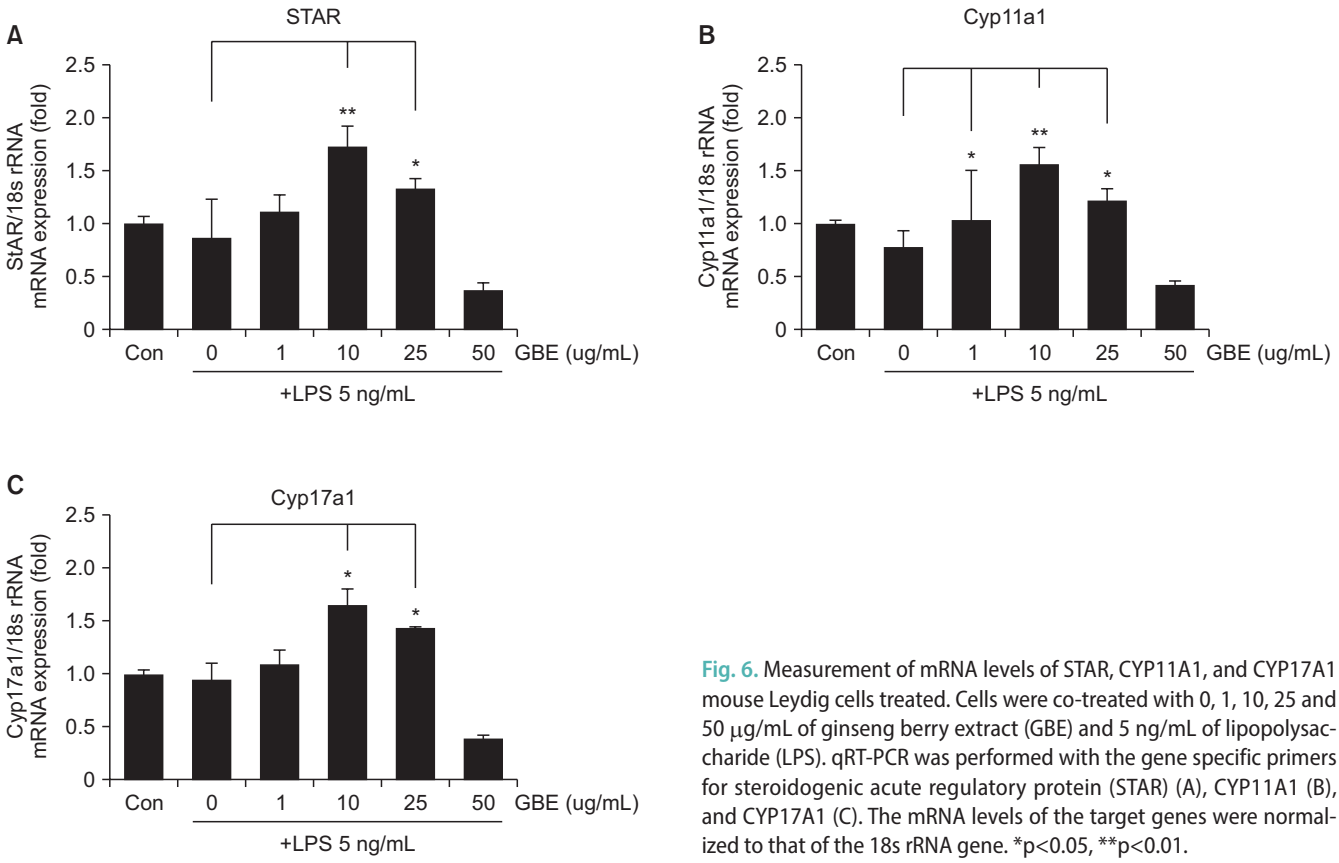

Fig. 6. Measurement of mRNA levels of STAR, CYP11A1, and CYP17A1 mouse Leydig cells treated. Cells were co-treated with 0, 1, 10, 25 and 50 μg/mL of ginseng berry extract (GBE) and 5 ng/mL of lipopolysaccharide (LPS). qRT-PCR was performed with the gene specific primers for steroidogenic acute regulatory protein (STAR) (A), CYP11A1 (B), and CYP17A1 (C). The mRNA levels of the target genes were normalized to that of the 18s rRNA gene. *p<0.05, **p<0.01.

steroidogenesis *in vitro*.

5. GBE induces mRNA expression of steroidogenesis-related genes in mice testes

To examine the steroidogenic effect of GBE *in vivo*, the expression of the steroidogenic genes were analyzed in the mice model. The animal experiment schedule described in Fig. 9. We dissected and harvest the mice testes for the further experiment. The mRNA levels of STAR, CYP11A1, and CYP17A1 were increased by GBE. In the LPS-injected group, the levels of these three genes were decreased compared to those in the GBE group. In particular, the expression of STAR, CYP11A1, and CYP17A1 increased in the 50 GBE group compared to the 100 mg/kg GBE group. (Fig. 10). This indicates that GBE is involved in male steroidogenesis by activating the steroidogenic enzymes, STAR, CYP11A1, and CYP17A1, at specific dosages (50 mg/kg) and increasing their mRNA levels *in vivo* (Fig. 10).

6. GBE increases the protein expression of steroidogenesis-related genes in the mice testes

Next, the effect of GBE on steroidogenic protein expression was evaluated *in vivo*. The protein levels of STAR, CYP11A1, and CYP17A1 were significantly increased by GBE (50 or 100 mg/kg) ingestion (Fig. 11). This indicates that GBE activated the steroidogenic enzymes, STAR, CYP11A1, and CYP17A1 and increased their protein levels *in vivo* in the mouse testis tissues (Fig. 11).

7. GBE enhances testosterone synthesis in the mice blood serum

After investigated the mRNA and protein levels of the steroidogenic genes in the mice testis tissues, we measured the synthetic testosterone levels in the mice blood serum. The testosterone levels were significantly increased in the GBE-fed group (50 or 100 mg/kg) when compared to the LPS-injected group (Fig. 12). Thus, GBE also enhanced the testosterone levels in the mice blood serum level directly, highlighting its involvement in the synthesis of testosterone *in vivo*.

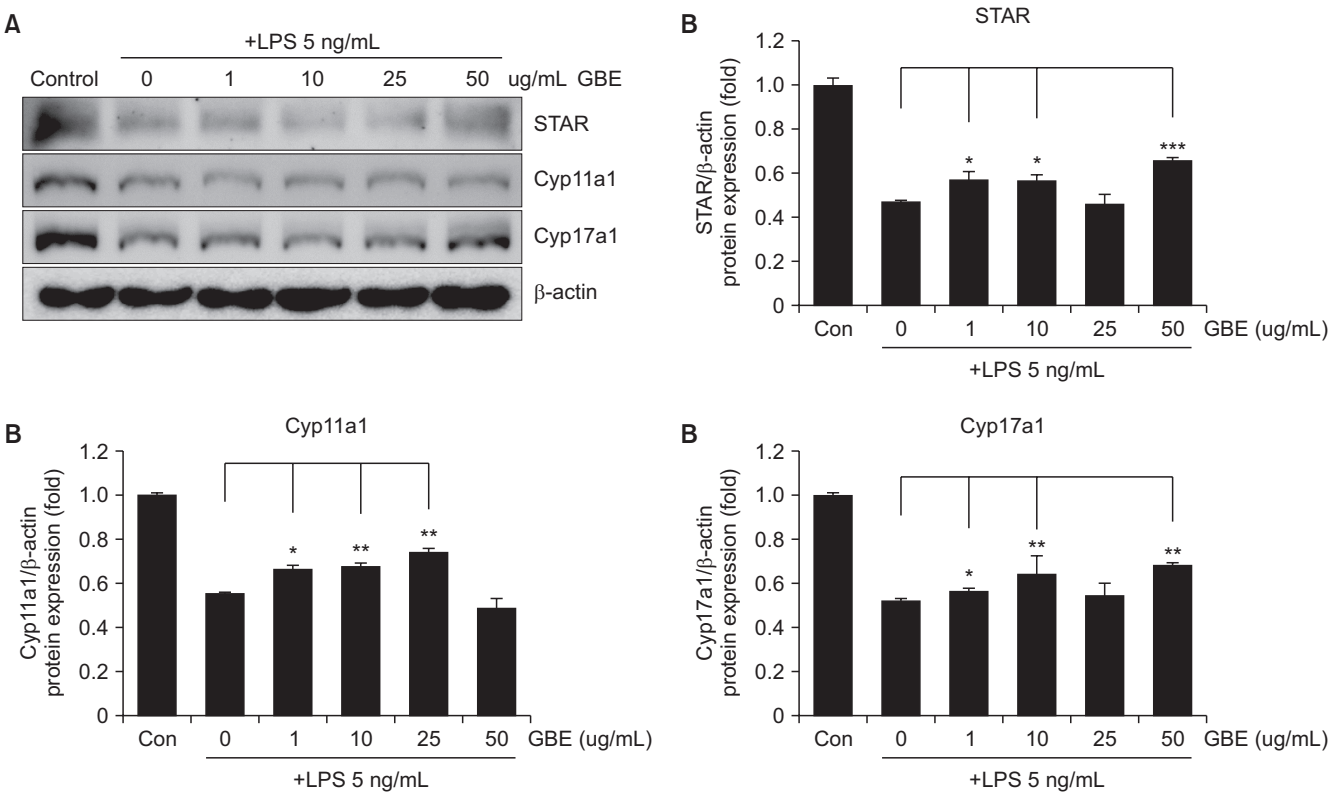

Fig. 7. Measurement of protein levels of steroidogenic acute regulatory protein (STAR), CYP11A1, and CYP17A1 in mouse Leydig cells. Cells were co-treated with 0, 1, 10, 25, and 50 μg/mL of ginseng berry extract (GBE) and 5 ng/mL of lipopolysaccharide (LPS). Western blotting was performed with the gene specific antibodies for STAR, CYP11A1, CYP17A1, and β-ACTIN (A). Total protein was normalized to β-ACTIN expression (B-D). *p<0.05, **p<0.01, ***p<0.001.

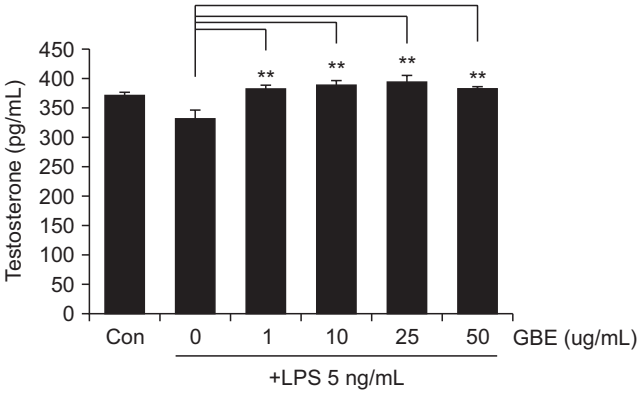

Fig. 8. Measurement of testosterone levels in mouse Leydig cell supernatants. Cells were treated with 0, 1, 10, 25, and 50 μg/mL of ginseng berry extract (GBE) and 5 ng/mL of lipopolysaccharide (LPS). The cell supernatant was collected for ELISA. **p<0.01.

8. GBE increases the mRNA expression of steroidogenesis-related genes in the testes of sleep deprived mice

To further confirm, we performed a sleep deprivation study on mice, with or without GBE treatment. The animal experimental schedule is shown in Fig. 13A. Photograph were taken at the beginning of the experiment (Fig. 13B). Mice were fed with 50 mg/kg/day of GBE for five days and were subjected to sleep deprivation for two days before sacrifice. We counted the number of mice that survived sleep deprivation. We observed that, in the 50 mg/kg/day GBE group, approximately 65% of the mice survived when compared to those in the sleep deprived group not fed GBE (Fig. 14). Next, the testes from the surviving mice were isolated and subjected to qRT-PCR to measure the expression of the steroidogenic genes, STAR, CYP11A1, and CYP17A1. The mRNA expressions of these genes were increased in the 50 mg/kg/day GBE group compared to those in the sleep deprived group not fed GBE (Fig. 15). We showed that GBE activated the steroidogenic enzymes and increased their mRNA levels *in vivo* in sleep deprived conditions. Thus, GBE has a steroidogenic effect in sleep deprived mice (Fig. 15).

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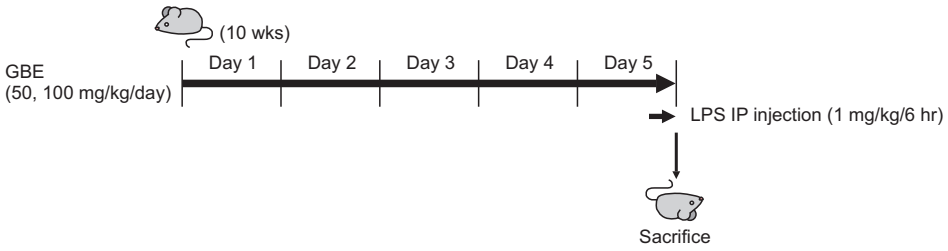

Fig. 9. The animal experiment schedule for ginseng berry extract (GBE) treatment and lipopolysaccharide (LPS) intraperitoneal (IP) injection. The mice were fed different concentrations (50 and 100 mg/kg/day) of GBE for 5 days.

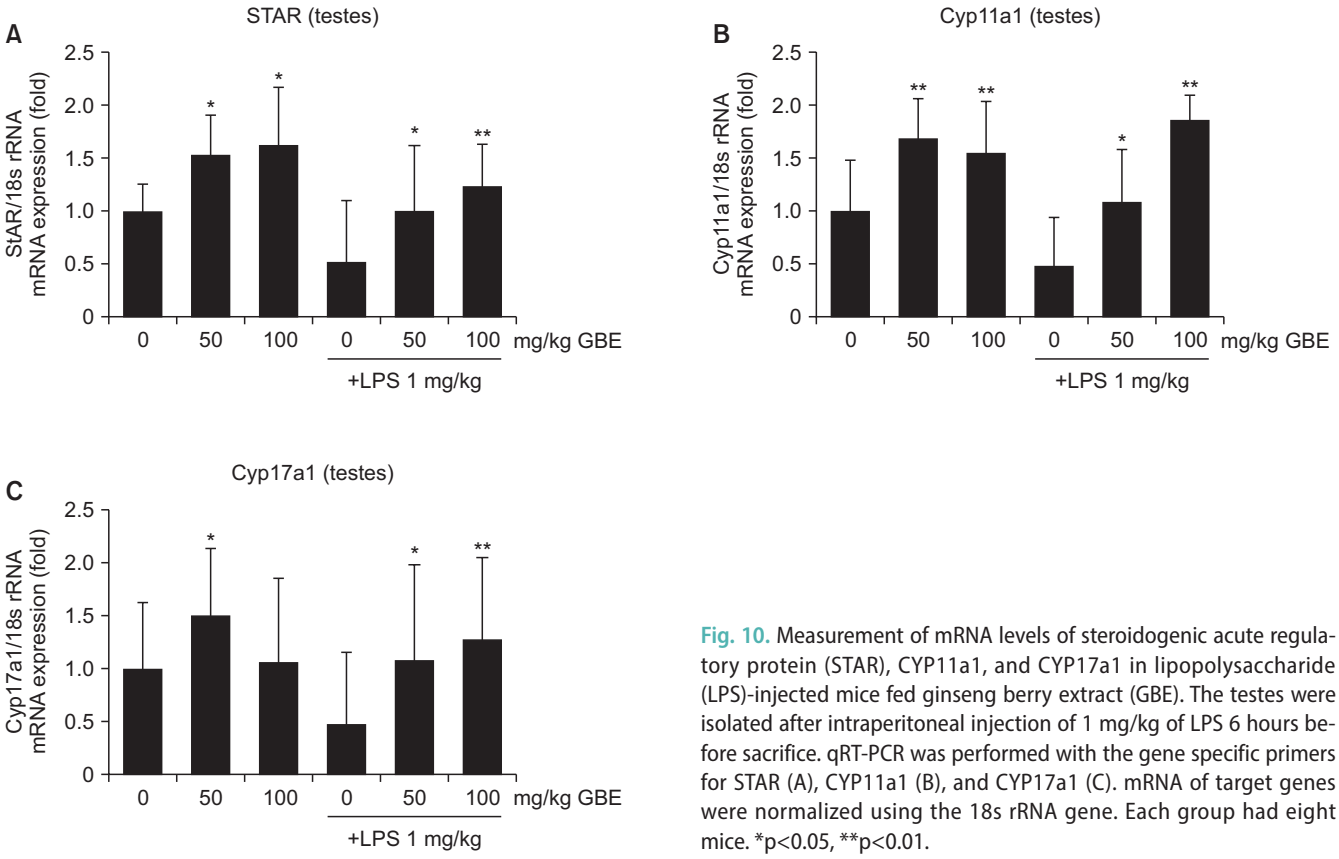

Fig. 10. Measurement of mRNA levels of steroidogenic acute regulatory protein (STAR), CYP11a1, and CYP17a1 in lipopolysaccharide (LPS)-injected mice fed ginseng berry extract (GBE). The testes were isolated after intraperitoneal injection of 1 mg/kg of LPS 6 hours before sacrifice. qRT-PCR was performed with the gene specific primers for STAR (A), CYP11a1 (B), and CYP17a1 (C). mRNA of target genes were normalized using the 18s rRNA gene. Each group had eight mice. *p<0.05, **p<0.01.

DISCUSSION

LOH is a vaguely defined disorder, but is nevertheless self-evident in its presence. While difficult to properly identify in its symptomatic context, it is generally defined as 'loss of vigor', often categorized by descriptive axes of sexual function, social activity, and physical robustness [17,22]. However, serum testosterone levels and its production have long been considered its central thesis of definition [15]. Studies defining LOH generally attempt to associate the various symptoms to serum testosterone levels, but with little success, as more often than not too many variables dilute the sensitivity of the questionnaires [22]. This may be due to the pervasive thinking that testosterone is primarily a sex hormone [23].

Hence, the current go-to remedy for LOH is immediate testosterone replacement by the clinician when the subject patient professes symptoms of weakness, decreased libido, fatigue, and shortened capacity for physical and mental exertion. Unfortunately, simply supplying testosterone in its raw form, despite advances to make it safer, presents the risk of unbalancing normal hormone-dependent behaviors; the risks also include development of malignancies [24]. With this caveat in mind, it is not considered safe to indefinitely resort to testosterone replacement. Hence, a natural supplement that could reverse the seemingly inevitable decline in testosterone production remains a valid and active enterprise. Despite its preconceived prejudicial

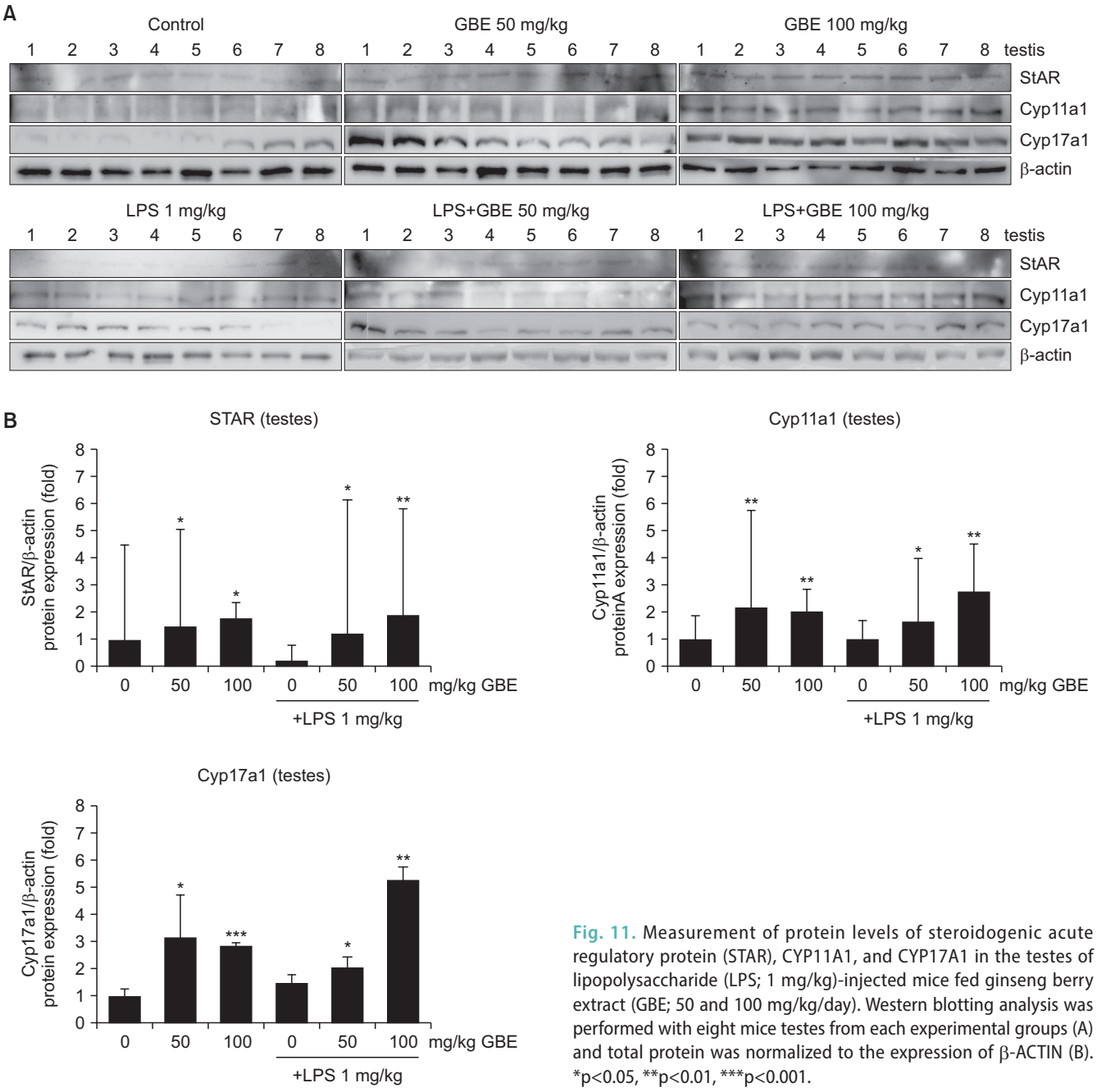

Fig. 11. Measurement of protein levels of steroidogenic acute regulatory protein (STAR), CYP11A1, and CYP17A1 in the testes of lipopolysaccharide (LPS; 1 mg/kg)-injected mice fed ginseng berry extract (GBE; 50 and 100 mg/kg/day). Western blotting analysis was performed with eight mice testes from each experimental groups (A) and total protein was normalized to the expression of β-ACTIN (B). *p<0.05, **p<0.01, ***p<0.001.

role as a male sex hormone, testosterone has recently been highlighted as a stress modulator, an immune modulator, and consequently in its inevitable sexual role, as a decisive factor which determines when a biological entity is fit for mating, viz a viz solitary survival [25,26]. Therefore, the investigation for a safe and sustainable, yet indirect, source of testosterone could be pivotal to longevity and natural immunity.

The current study demonstrates the effects of the GBE on testosterone production, *in vitro* and *in vivo*. This was demonstrated by analyzing TM3 cell cultures, to assessing the survivability and serum production in the living animal, and finally, evaluating the survivability and health of the animal under acute stress situations. This is a multidimensional display of the effects of GBE, although not directly conductive to the next dimensionality.

This study shows direct evidence that GBE improves testosterone production via upregulation of steroidogenic genes, STAR, CYP11A1, and CYP17A1, at both the mRNA and protein levels. However, these effects on TM3 cells were not associated indirectly with other

metabolic or vascular effects, such as diabetes, obesity, or radical oxygen species [2,7,14,27].

Our results of the effects of GBE in improving steroidogenesis or LOH were consistent with those obtained using other medicinal plants. We already had investigated Korean medicinal plants for improving LOH with TOE [19] and Korean rice bran extract (*Oryza sativa L.*) [20] by increasing steroidogenesis-related enzymes (*i.e.*, StAR, CYP11A1, and CYP17A1), which facilitate the production of testosterone in TM3 mouse Leydig cells and mice testis (Fig. 16).

The results showed that GBE administration improved mouse serum testosterone levels. This could have been explained by a plethora of alternate processes. However, given that the androgenic steroid production increased on the cellular level, along with the direct evidence of the improvement in testes volume, it can be confirmed that GBE improves serum testosterone levels.

Currently, there is no definitive mouse or *in vivo* model of LOH. LOH is vaguely defined in humans, and it requires social and behavioral estimates for a proper assessment in contrast to its similar but endocrinologically defined sister concept, testosterone deficiency syndrome [15]. Additionally, LOH, unlike hypogonadism due to other causes, is a disorder of aging, by definition. However, similar *in vivo* models have equated aging with stress burden; in our study middle-aged or older mice were exposed to acute mental and physical stress of paradoxical sleep deprivation through a modified muti-platform method, previously defined for rat [28]. Furthermore, this model's effect on serum testosterone levels and hypogonadism has previously been demonstrated [21].

The model successfully applied physical and mental stress to the mice, and the decrease in testosterone level has also been demonstrated independently in mice of the same cohort, as well as in mice under stress. As such, the model presents a verisimilitude of LOH in mice. In this study, these mice showed drastic difference in survivability, as not only improved serum testosterone levels and body weight and size that differed between groups, but also improved survivability of the organism under severe stress.

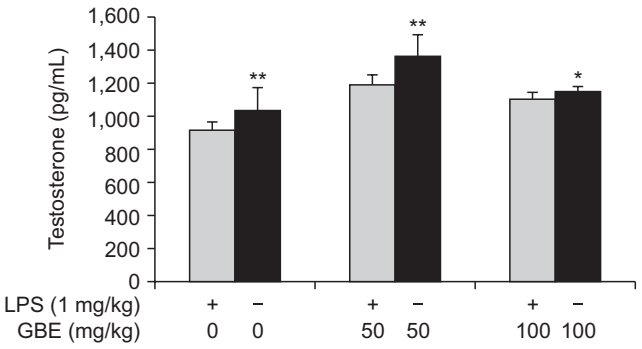

Fig. 12. Measurement testosterone levels in the mouse blood serum. Mice were fed 50 and 100 mg/kg/day of ginseng berry extract (GBE), with or without lipopolysaccharide (LPS) injection (1 mg/kg/day). The blood serum were collected and ELISA was performed. *p<0.05, **p<0.01. Each experimental group contained eight mice blood samples.

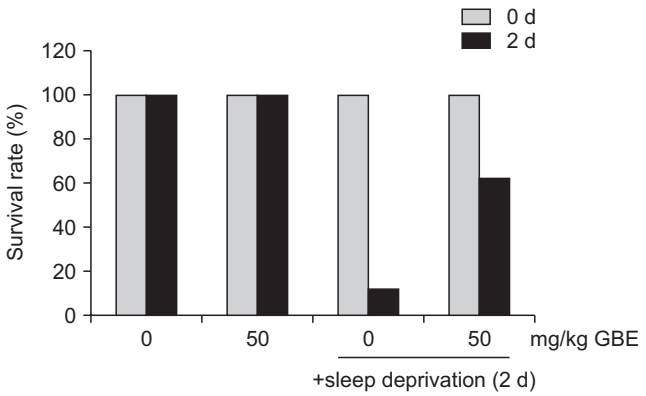

Fig. 14. Measurement survival in sleep deprived mice. Mice were fed with 50 mg/kg/day of ginseng berry extract (GBE), with or without sleep deprivation. Each group had eight mice. The survival rate was measured after two days of sleep deprivation.

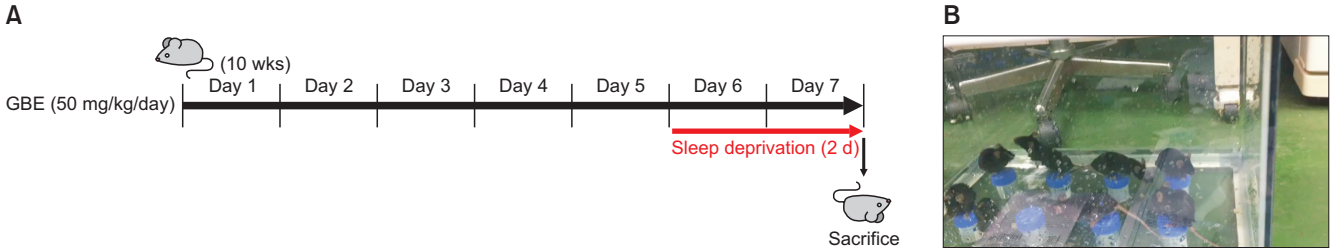

Fig. 13. The animal experiment schedule for sleep deprivation. The different concentrations (50 and 100 mg/kg/day) of GBE were fed to the mice for 7 days (A). The picture of sleep deprivation was taken at the beginning of the experiment (B).

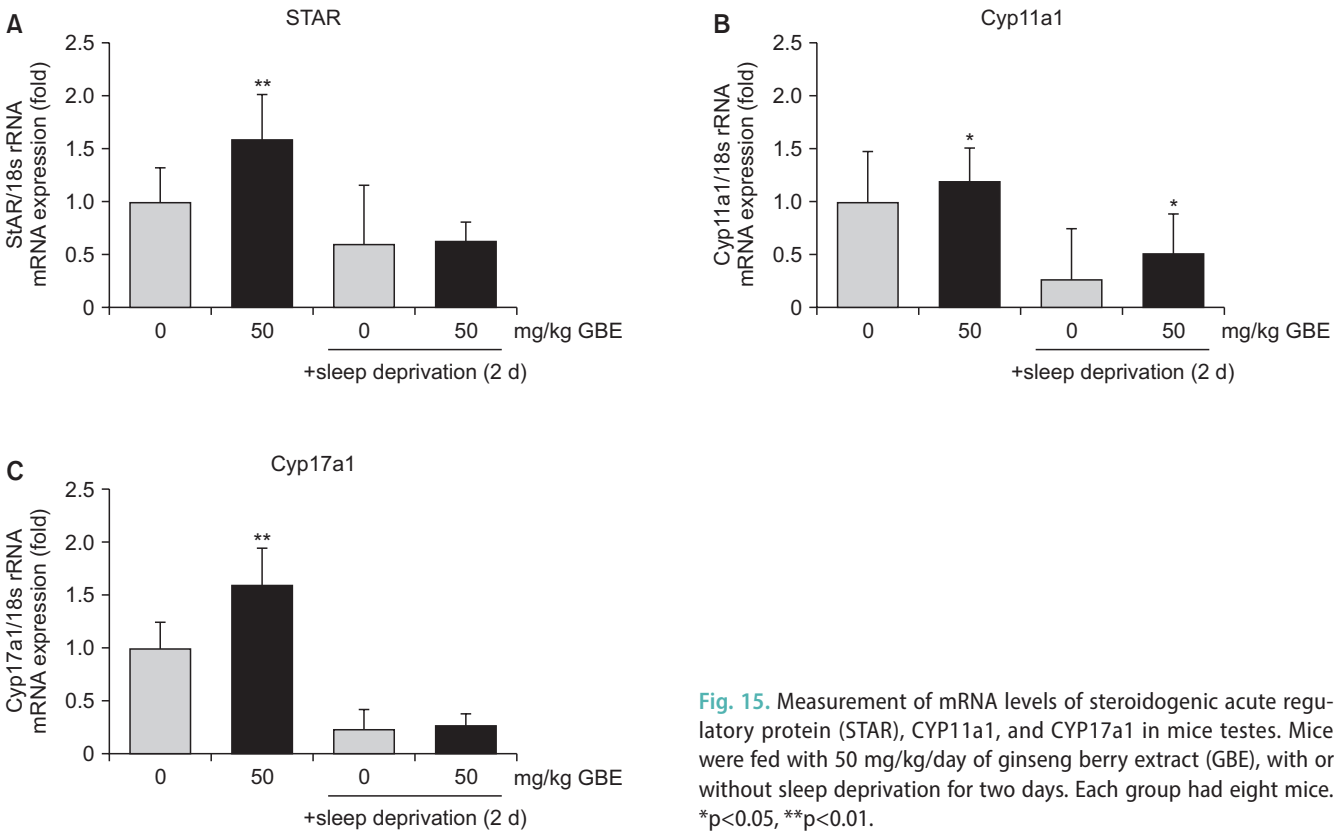

Fig. 15. Measurement of mRNA levels of steroidogenic acute regulatory protein (STAR), CYP11a1, and CYP17a1 in mice testes. Mice were fed with 50 mg/kg/day of ginseng berry extract (GBE), with or without sleep deprivation for two days. Each group had eight mice. *p<0.05, **p<0.01.

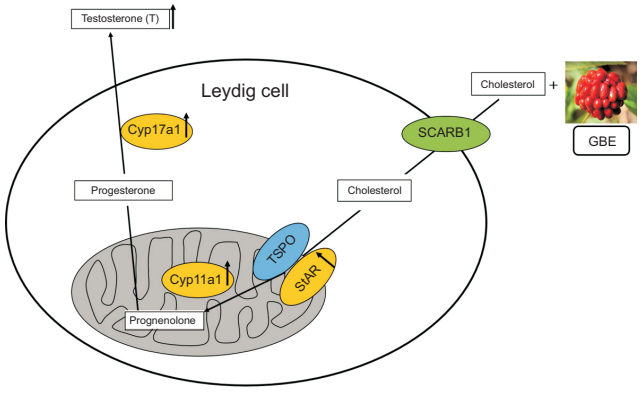

Fig. 16. Steroidogenesis in Leydig cells. The hypothesis in this study were schematized. SCARB1: scavenger receptor class B member1, TSPO: translocator protein, GBE: ginseng berry extract, STAR: steroidogenic acute regulatory protein, CYP11A1: cytochrome P450 family 11 subfamily A member 1, CYP17A1: cytochrome P450 family 17 subfamily A member 1.

This study has some drawbacks. Like any study investigating the properties of natural extracts, the current study suffers from ill-defined combinations of effective compounds. However, the ginseng berry purification method is not a novel concept, and the extraction methods used in this study are well-documented and comparable to those reported in literature [7,10,12,29].

The current study has primarily been focused on direct application of the extract on the mice model. While we have performed extensive investigation with, albeit, promising results, the study is still essentially a pre-clinical study. Further cautious application with standardization methods and rigorous safety analysis is required for full applicability for human clinical trials. Preparations for clinical trials of the extraction are underway. While mice and men are not directly scalable, further investigations on the effective compounds and the mechanisms through which Leydig cells were preserved and/or recovered, would help shed light on this subject.

CONCLUSIONS

We demonstrated that the Korean GBE increased both mRNA and protein levels of steroidogenesis-related enzymes STAR, CYP11A1, and CYP17A1 both *in vitro* and *in vivo*. These key enzymes induce the production of testosterone in mouse Leydig cells as well as in mice testes. This study shows that GBE may be used an additives with food or medicine for the treatment

of diseases characterized by insufficient testosterone, including male infertility, hypogonadism, and LOH. However, further studies are needed to establish the clinical efficacy of GBE. From our results, it can be concluded that GBE is a potential, promising therapeutic agent for men's health that increases steroidogenesis or improves LOH.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: JWK, SCM, SJL. Data curation: JWK, HJC. Formal analysis: JWK, HJC. Funding acquisition: SJL. Investigation: HJC, SJL. Methodology: HJC, JWK, AJ, CEL, DWL. Project administration: JWK, SCM. Resources: JWK, SCM, SJL. Software: JWK, HJC. Supervision: SJL. Validation: JWK, HJC. Visualization: HJC. Writing – original draft: JWK, SCM. Writing – review & editing: JWK, HJC.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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