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Development of a Long-Acting Follicle-Stimulating Hormone Using Serum Albumin Fab-Associated Technology for Female Infertility

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Background: Recombinant human follicle-stimulating hormone (rhFSH) is commonly used to treat female infertility, but its short half-life necessitates multiple doses. Even corifollitropin alfa, with an extended half-life, requires supplementary injections of rhFSH after 7 days. This study aimed to develop and evaluate a long-acting follicle-stimulating hormone (FSH) formulation using anti-serum albumin Fab-associated (SAFA) technology to avoid additional injections and enhance ovarian function.

Methods: SAFA-FSH was synthesized using a Chinese hamster ovary expression system. Its biological efficacy was confirmed through assays measuring its ability to stimulate cyclic adenosine monophosphate (cAMP) production, estradiol synthesis, and the expression of human cytochrome P450 family 19 subfamily A member 1 (hCYP19α1) and human steroidogenic acute regulatory protein (hSTAR) in human ovarian granulosa (KGN) cells. To evaluate the effects of SAFA-FSH, we compared its impact on serum estradiol levels and ovarian weight increase with that of rhFSH in Sprague-Dawley (SD) rats using the modified Steelman-Pohley test.

Results: The results indicated that SAFA-FSH induces cAMP synthesis in KGN cells and upregulates the expression of hCYP19α1 and hSTAR in a dose-dependent manner. Female SD rats, aged 21 days, receiving daily subcutaneous human chorionic gonadotropin injections for 5 days exhibited a significant increase in serum estradiol levels and ovarian weight when administered SAFA-FSH on the first day or when given nine injections of rhFSH over 5 days. Notably, the group receiving SAFA-FSH on the first and third days demonstrated an even greater rise in serum estradiol levels and ovarian weight.

Conclusion: These findings suggest that SAFA-FSH presents a promising alternative to current rhFSH treatments for female infertility. However, further research is essential to thoroughly assess its safety and efficacy in clinical contexts.

Keywords: Follicle stimulating hormone; Female infertility; Ovulation; Gonadotropins

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INTRODUCTION

Infertility is defined as the inability to achieve pregnancy after 12 months of regular, unprotected sexual intercourse [1]. According to a study conducted by the World Health Organization involving 8,500 infertile couples, female factor infertility was reported in 37% of couples in developed countries [2]. Identifiable female factors contributing to infertility include ovulatory disorders, endometriosis, pelvic adhesions, and related conditions. Ovulatory disorders are discernible in 18% to 25% of couples experiencing infertility [3-5].

Gonadotropin therapy plays a pivotal role in the standard treatment of infertility in both assisted reproductive technology (ART) and non-ART cycles [6]. Recombinant human follicle-stimulating hormone (rhFSH) preparations, due to their short serum half-life and rapid metabolic clearance, necessitate daily injections to maintain follicle-stimulating hormone (FSH) levels above the threshold during ovarian stimulation. However, frequent injections can elevate stress levels and increase the likelihood of errors. Consequently, ovarian stimulation protocols requiring fewer injections have the potential to enhance adherence, reduce errors, and decrease financial costs [7].

Corifollitropin alfa, a clinically available long-acting FSH, is utilized for controlled ovarian stimulation in conjunction with a gonadotropin-releasing hormone (GnRH) antagonist to foster the development of multiple follicles in women undergoing fertility treatment [8]. Despite corifollitropin alfa boasting a half-life of 7 days post-injection, daily rhFSH injections must persist until meeting the criteria for triggering final oocyte maturation in women.

This study investigates the potential of anti-serum albumin Fab-associated (SAFA) technology to formulate a long-acting FSH that obviates the need for additional injections. Our research aims to characterize the bioactivity of SAFA-FSH compared to that of rhFSH both *in vitro* and *in vivo*.

METHODS

Reagents

SAFA-FSH was synthesized according to previously established methods by AprilBio Co. Ltd. (Chuncheon, Korea) [9]. An illustration of SAFA-FSH is shown in Fig. 1A. The binding affinity of SAFA-FSH to human serum albumin is similar to that of SL335 (SAFA body), and its half-maximal effective concentration (EC50) is approximately 479 pM (Fig. 1B). The sequence-based molecular weight of SAFA-FSH is 71 kDa, with four N-linked glycosylation sites. It was measured to be approximately 80 kDa using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) (Fig. 1C). rhFSH (follitrope) and human chorionic gonadotropin (hCG; IVF-C) were procured from LG Chem (Seoul, Korea).

Cell culture

A human ovarian granulosa (KGN) cell line (RCB1154, RIKEN BioResource Research Center, Kyoto, Japan), were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Hyclone) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

Measurement of cyclic adenosine monophosphate production

KGN cells were seeded at a density of 0.5×10^6 cells/plate and cultured in 6-cm plates. Following a 4-hour period of starvation, the cells were treated with varying concentrations of rhFSH or SAFA-FSH in the presence of 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO, USA) for 6 hours at 37°C. Subsequently, the supernatant was collected, and cyclic adenosine monophosphate (cAMP) concentrations were determined using a cAMP XP assay kit (Cell Signaling Technology, Danvers, MA, USA).

Estradiol measurement

KGN cells were seeded at a density of 0.5×10^6 cells per plate and cultured in 6-cm plates. Following an overnight period of starvation, the cells were exposed to varying concentrations of rhFSH or SAFA-FSH in the presence of 100 μM androstenedione (Sigma-Aldrich) for 48 hours at 37°C. The supernatant from the stimulated KGN cells was collected, and estradiol levels were quantified using an Estradiol Parameter Assay Kit (Cusabio, Wuhan, China) following the manufacturer's instructions.

RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated from cell lines using the TRIzol reagent (Invitrogen, MA, USA), and cDNA was synthesized from 2 μg of total RNA employing the ReverTra Ace quantitative polymerase chain reaction (qPCR) RT Kit (Toyobo, Osaka, Japan). The following primer sequences were utilized: 5′-TGGAAATGCTGAACCCGATAC-3′ (sense) and 5′-AATTCCCATGCAGTAGCCAGG-3′ (antisense) for human cytochrome P450 family

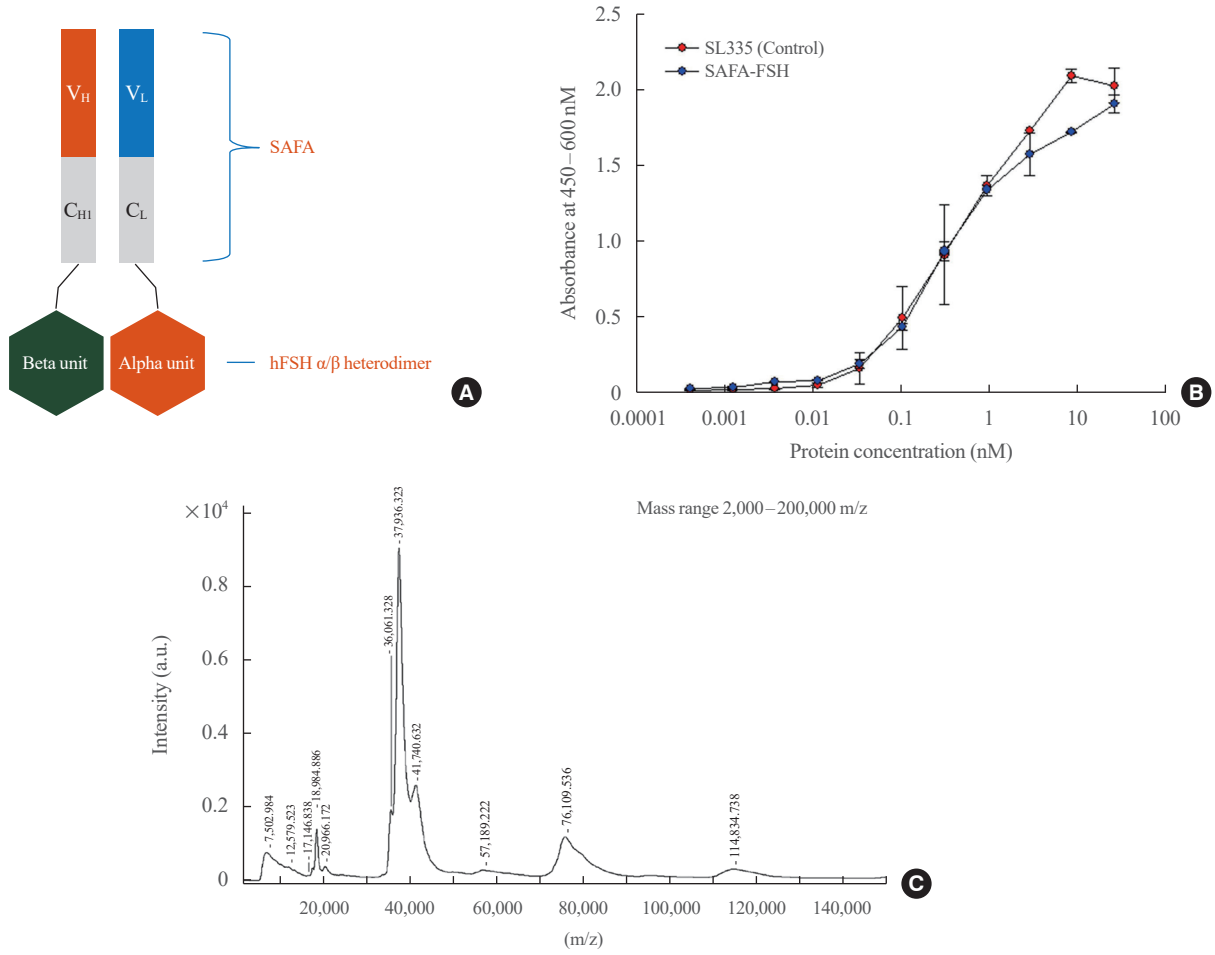

Fig. 1. The characteristics of anti-serum albumin Fab-associated (SAFA)-follicle-stimulating hormone (FSH). (A) SAFA-FSH consists of alpha and beta subunits, (B) human serum albumin binding capacity of SAFA-FSH, and (C) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis results of SAFA-FSH. hFSH, human follicle-stimulating hormone; a.u., arbitrary unit.

19 subfamily A member 1 (hCYP19α1); 5′-GGGAGTGGAACCCCAATGTC-3′ (sense) and 5′-CCAGCTCGTGAGTAATGAATGT-3′ (antisense) for human steroidogenic acute regulatory protein (hSTAR); and 5′-GGAGCGAGATCCCTCCAAAAT-3′ (sense) and 5′-GGCTGTTGTCATACTTCTCATGG-3′ (antisense) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). qPCR reactions comprised 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 5 pmol of each forward and reverse primer, 2 μL of diluted cDNA template, and sterile distilled water to achieve a final volume of 20 μL. PCR was conducted on an ABI StepOnePlus Real Time PCR system (Applied Biosystems) under the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative quantification was performed using the comparative cycle threshold method, with GAPDH serving as an internal control.

Animal experimental design

Female Sprague-Dawley (SD) rats were procured from Orient Bio (Seoul, Korea) and housed under controlled conditions (22°C, 12-hour light-dark cycle) with access to rodent chow and tap water *ad libitum*. The rats were allowed to acclimate for 1 week before the commencement of the study. Three-week-old and 50 g female SD rats were randomly assigned to four groups (n=5 per group) (Fig. 2A). All groups, including the control group, received daily subcutaneous injections of hCG (13 IU) throughout the experiment [10]. The rhFSH group received nine subcutaneous injections of rhFSH (4 μg/kg) every 12 hours over a 5-day period. The SAFA-FSH I group received subcutaneous injections of SAFA-FSH (21.3 μg/kg, three times the molar amount of rhFSH considering the molecular weight) every 12 hours on the first day. Conversely, the SAFA-FSH II group received a subcutaneous injection of SAFA-FSH (21.3 μg/kg) ev-

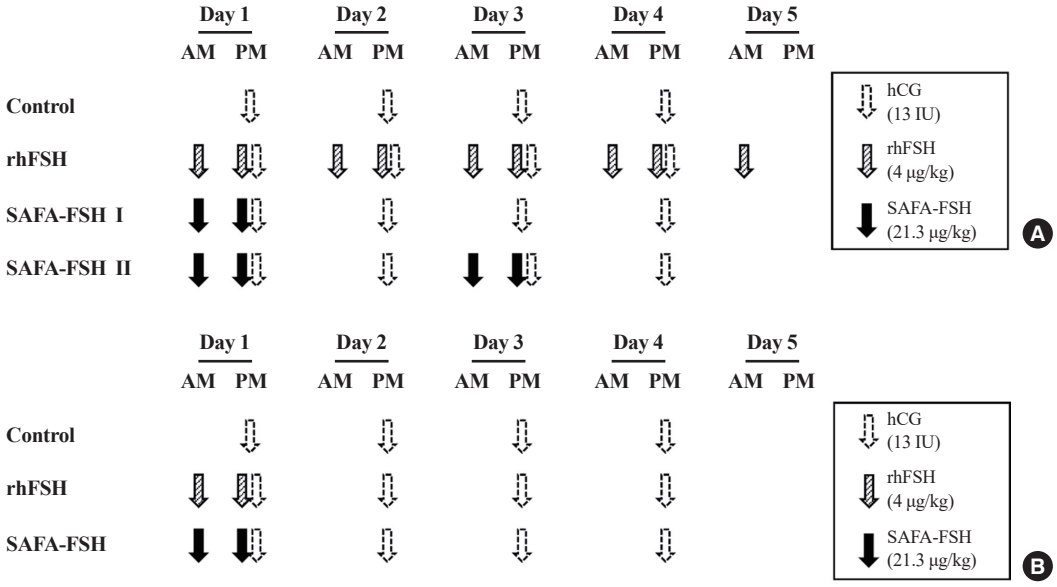

Fig. 2. Ovulation induction time course with anti-serum albumin Fab-associated (SAFA)-follicle-stimulating hormone (FSH) in Sprague-Dawley rats. (A) First experiment, (B) second experiment. Arrows indicate the injection cycle of each drug. Open arrows represent human chorionic gonadotropin (hCG) injections, shaded arrows represent recombinant human follicle-stimulating hormone (rhFSH) injections, and closed arrows represent SAFA-FSH injections.

ery 12 hours on the first and third days. The dosage of SAFA-FSH was determined based on differences in bioactivity and molecular weight compared to rhFSH, informed by this study and previous *in vitro* experiments [9]. Body weights were recorded before euthanasia, and the rats were euthanized on the afternoon of the fifth day.

In a second experiment, female SD rats (3-week-old and weighing 50 g) were randomly allocated to three groups (n=6–7 per group) (Fig. 2B). Similar to the first experiment, all groups received daily subcutaneous injections of hCG (13 IU) throughout the study period. The rhFSH and SAFA-FSH groups were administered subcutaneous injections of rhFSH (4 μg/kg) or SAFA-FSH (21.3 μg/kg) every 12 hours on the first day, with subsequent procedures conducted analogously to the first experiment. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System under approval number 2021-0087.

Serum estradiol measurement and ovary assessment

Blood samples were collected from the orbital sinus using capillary tubes, and serum was isolated by centrifugation at 3,000 rpm for 10 minutes at 4°C. Serum estradiol levels were determined using an Estradiol Parameter Assay Kit (Cusabio) following the manufacturer's instructions. Ovaries were excised, weighed, and subjected to hematoxylin and eosin staining for quantitative assessment of follicle morphology and count.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 25 (IBM, Armonk, NY, USA). Data are presented as mean±standard error of the mean. Statistical significance was assessed using an unpaired t test, with differences considered statistically significant at P<0.05.

RESURTS

In vitro functional assays

The ability of SAFA-FSH to activate the FSH receptor upon binding was assessed by measuring cAMP production (Fig. 3A). KGN cells were exposed to various concentrations of rhFSH or SAFA-FSH. Although SAFA-FSH exhibited slightly lower potency compared to rhFSH, it effectively stimulated cAMP synthesis in KGN cells.

Evaluating estradiol synthesis, KGN cells treated with rhFSH or SAFA-FSH showed a dose-dependent increase in estradiol production (Fig. 3B). Notably, estradiol production at 100 nM rhFSH was comparable to that of SAFA-FSH within the concentration range of 250 to 500 nM.

Assessment of aromatase expression in KGN cells treated with rhFSH or SAFA-FSH revealed intriguing findings (Fig.

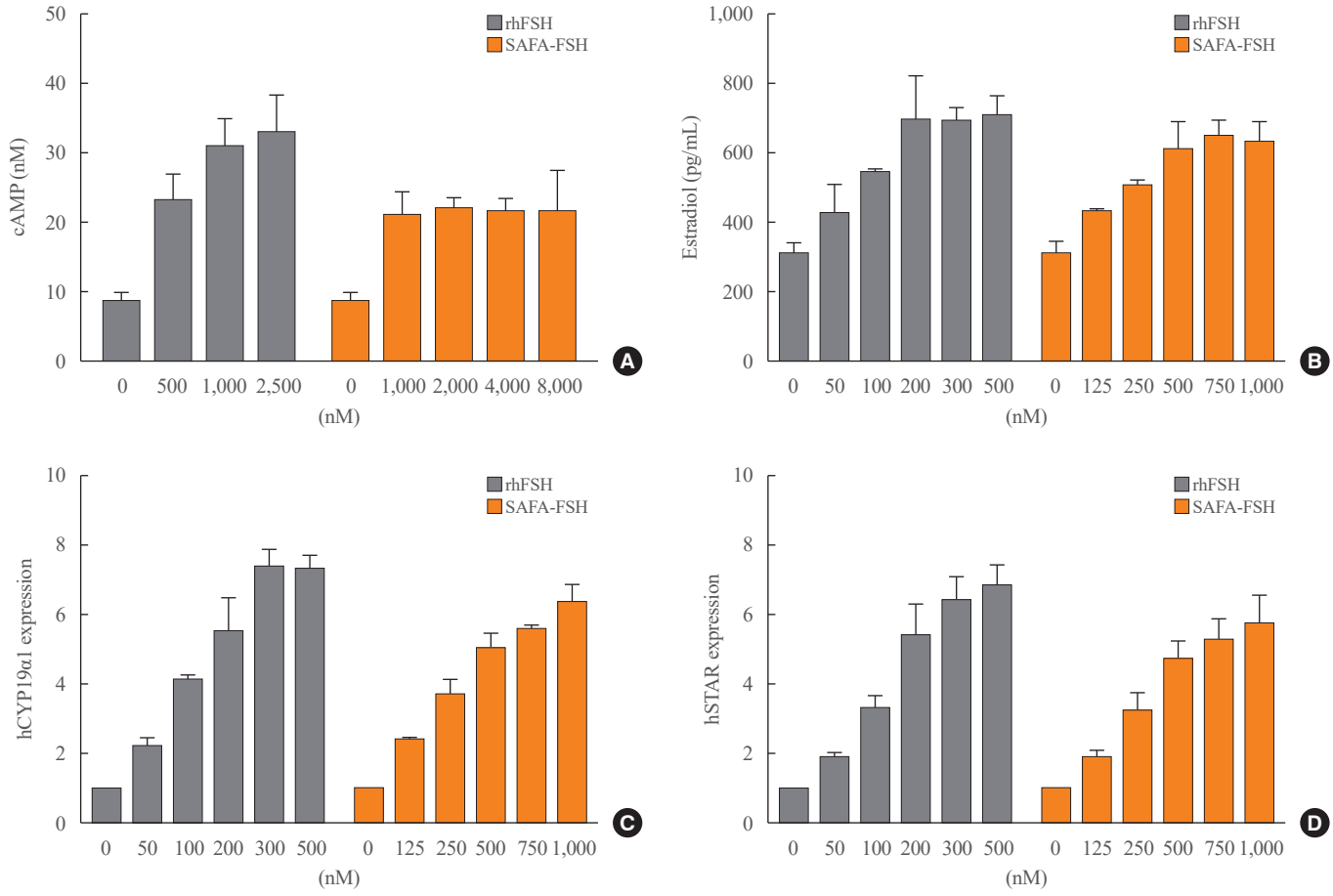

Fig. 3. Effects of anti-serum albumin Fab-associated (SAFA)-follicle-stimulating hormone (FSH) in cell line experiments. (A) Cyclic adenosine monophosphate (cAMP) production, (B) estradiol synthesis, (C) human cytochrome P450 family 19 subfamily A member 1 (hCYP19α1) expression, and (D) human steroidogenic acute regulatory protein (hSTAR) expression in human ovarian granulosa (KGN) cells treated with indicated doses of recombinant human follicle-stimulating hormone (rhFSH) or SAFA-FSH. Data are representative of three independent experiments and are presented as mean±standard error of the mean.

3C, D). The expression of the aromatase enzyme hCYP19α1 increased with increasing concentrations of SAFA-FSH, with similar expression levels noted between 250 and 500 nM of SAFA-FSH compared to rhFSH at 100 nM. Moreover, the expression of hSTAR increased with increasing concentrations of SAFA-FSH, showing comparable levels between 250 nM SAFA-FSH and 100 nM rhFSH.

In vivo functional assays

To validate the prolonged action of SAFA-FSH as a hormone, rats received treatment with either rhFSH or SAFA-FSH, alongside daily hCG injections. At the study's conclusion, no discernible difference in body weight was noted among the groups (Fig. 4A).

Regarding serum estradiol levels, both the rhFSH group and SAFA-FSH I group exhibited a significant increase compared to the control group (P=0.038 and P=0.029, respectively) (Fig. 4B). Furthermore, the SAFA-FSH II group demonstrated a significant elevation in serum estradiol levels compared with both the control group (P=0.001) and the rhFSH group (P=0.014).

In the experimental groups, ovarian weight significantly exceeded that of the control group (right: P=0.008, P=0.002, and P<0.001; left: P=0.008, P<0.001, and P<0.001, respectively) (Fig. 4C). The ovarian weight in the SAFA-FSH I group, administered on the first day, was comparable to that in the rhFSH group treated daily for 5 days, indicating the sustained effect of SAFA-FSH. Moreover, the SAFA-FSH II group, receiving treatment on the first and third days, exhibited even greater increases in ovarian weight compared to the control group.

When assessing follicles larger than 140 μm to gauge the drug

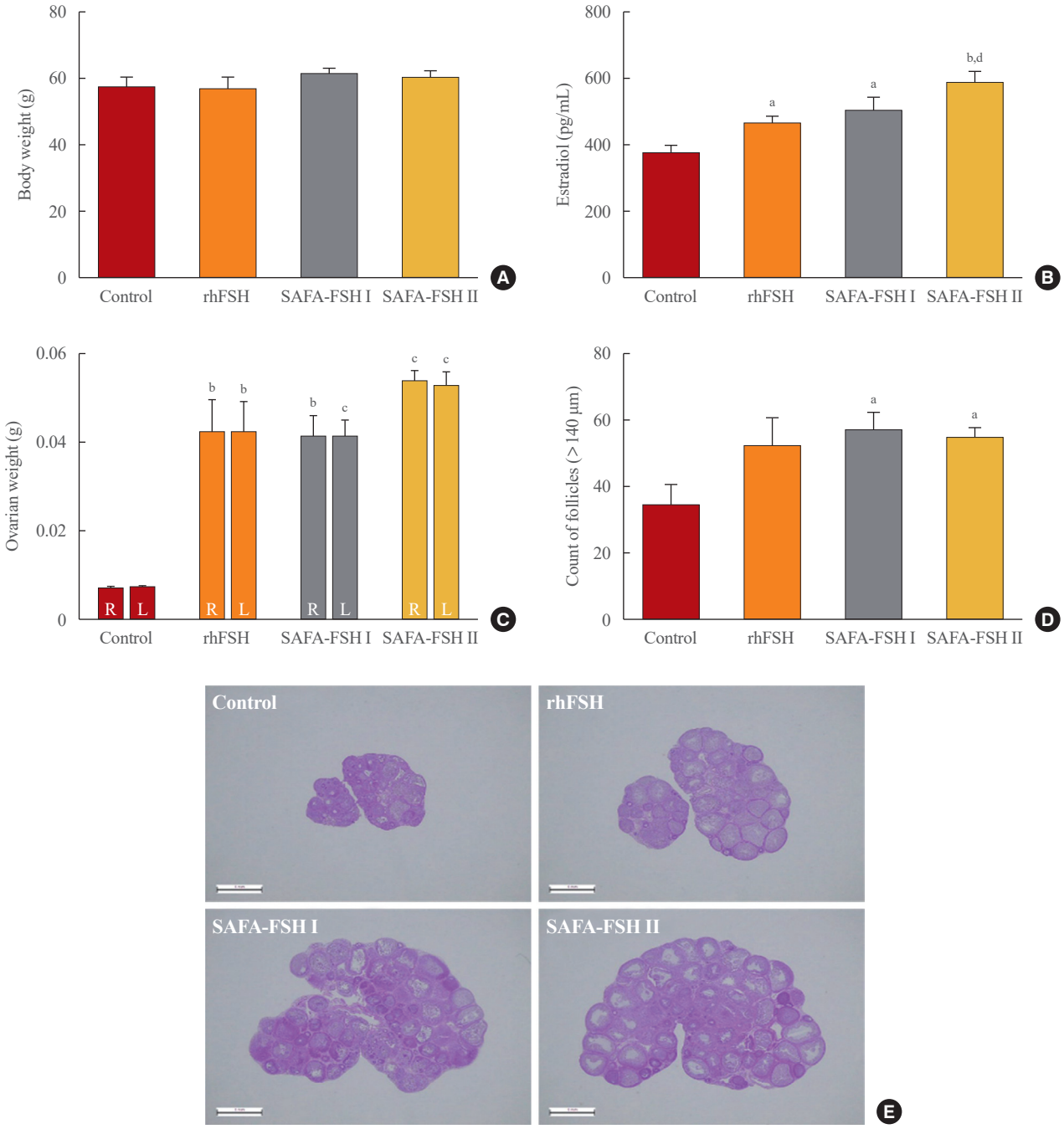

Fig. 4. Effects of anti-serum albumin Fab-associated (SAFA)-follicle-stimulating hormone (FSH) in experimental animals. (A) Body weight, (B) serum estradiol level, (C) ovarian weight, (D) follicle count (>140 μm), and (E) representative images of ovaries stained with hematoxylin and eosin (×4). Data are presented as mean±standard error of the mean. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ vs. control group; [d]$P<0.05$ vs. recombinant human follicle-stimulating hormone (rhFSH) group.

response, the rhFSH group showed a slight but statistically insignificant increase compared to the control group ($P=0.138$) (Fig. 4D). In contrast, both the SAFA-FSH I and SAFA-FSH II groups displayed notable and statistically significant increases compared to the control group ($P=0.030$ and $P=0.024$, respectively). Interestingly, in the SAFA-FSH II group, there appeared to be greater recruitment of early follicles despite a relatively lower number of antral follicles. Hematoxylin and eosin stain-

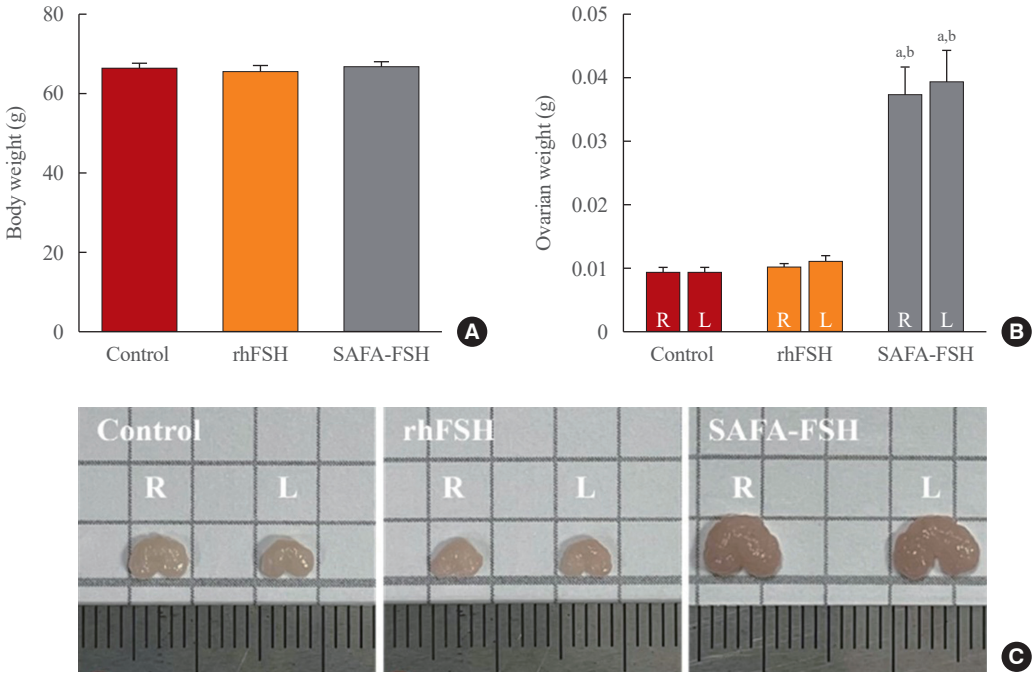

Fig. 5. Effects of anti-serum albumin Fab-associated (SAFA)-follicle-stimulating hormone (FSH) in the second set of experimental animals. (A) Body weight, (B) ovarian weight, and (C) representative images of ovaries. Data are presented as mean±standard error of the mean. [a]$P<0.01$ vs. control group; [b]$P<0.01$ vs. recombinant human follicle-stimulating hormone (rhFSH) group.

ing of ovarian sections also revealed significant follicle growth following rhFSH or SAFA-FSH treatment (Fig. 4E).

In a subsequent confirmatory experiment, rhFSH or SAFA-FSH was administered for only 1 day. No difference in body weight was noted among the groups (Fig. 5A). In the rhFSH group, ovarian weight did not increase significantly (right: $P=0.336$, left: $P=0.159$) (Fig. 5B). However, in the SAFA-FSH group, ovarian weight increased significantly compared to both the control and rhFSH groups (right: $P=0.001$, left: $P=0.001$ for both) (Fig. 5C). On visual observation, the ovaries in the SAFA-treated group were seen to be notably larger than those in the control and rhFSH groups. These results suggest that SAFA-FSH exhibits prolonged action, effectively increasing ovarian size and promoting follicle recruitment even with single-day administration, indicating its sustained effect.

DISCUSSION

In this study, we explored the potential of a long-acting FSH formulation to tackle the challenges associated with frequent injections in the treatment of female infertility. This formulation leverages SAFA technology and boasts a serum half-life of approximately 2 weeks in humans.

The KGN cell line, derived from ovarian granulosa cells, expresses the FSH receptor, leading to increased cAMP activity upon FSH stimulation, which facilitates the synthesis of estradiol from androstenedione by aromatase in theca cells [11]. Our study confirms that, akin to rhFSH, the administration of SAFA-FSH induces elevated cAMP levels and promotes estradiol synthesis in KGN cells. Furthermore, SAFA-FSH administration was found to upregulate the expression of aromatase enzymes hCYP19α1 and hSTAR, pivotal for estradiol synthesis. The bioactivity of SAFA-FSH appears to be approximately one-third that of rhFSH, as indicated by previous studies employing TM4 cells with overexpressed follicle-stimulating hormone receptor [9].

To assess the efficacy of SAFA-FSH as a long-acting hormone, *in vivo* functional assays were conducted. Rats received either rhFSH or SAFA-FSH, in addition to daily hCG injections across all groups, as part of the modified Steelman-Pohley test [10,12,13]. Notably, serum estradiol levels exhibited significant increases, particularly in the SAFA-FSH II group. SAFA-FSH treatment in rats led to augmented ovarian weight with sustained effects even after a single dose. Both SAFA-FSH groups demonstrated significant enhancements in follicle size compared to the control group. These findings underscore the poten-

tial of SAFA-FSH as a viable long-acting therapeutic option.

Corifollitropin alfa, the pioneer hybrid molecule demonstrating sustained follicle-stimulating effects, yielded notably high ongoing pregnancy rates comparable to those achieved with daily rhFSH [14]. Its protracted half-life enables it to supplant the initial seven injections of conventional gonadotropins during the fertility treatment cycle with a solitary injection. Nonetheless, daily rhFSH injections became imperative after 7 days to induce final oocyte maturation. Remarkably, in the randomized controlled trial of corifollitropin alfa, the maximum total stimulation duration was 19 days [14]. Although this study did not directly compare corifollitropin alfa and SAFA-FSH, corifollitropin alfa has a 1.5- to 2-fold longer serum half-life than rhFSH, whereas SAFA-FSH demonstrated a 2.7-fold increase in half-life compared to rhFSH in a previous rat study [9,10]. Despite the inevitable lower half-life in rats due to disparities in affinity and the shorter half-life of rat serum albumin, SAFA-FSH's half-life is anticipated to approximate 2 weeks in humans. Drawing from pharmacokinetic insights gleaned from a phase 1 clinical trial of APB-R3 (SAFA-IL-18BP), the drug's half-life was estimated to be 13 to 14 days. This indicates that a single injection of SAFA-FSH holds the potential to replace all gonadotropin injections administered during ovarian stimulation.

Prolonging the duration during which FSH levels remain above the threshold level can facilitate multiple ovulations [15]. However, ovarian stimulation may entail severe complications such as ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies. Employing the minimum dose and duration of gonadotropin therapy is crucial to achieving follicular development and pregnancy while mitigating the risk of OHSS, the most severe complication [16,17]. SAFA-FSH's anticipated lengthy half-life is expected to sustain the entire therapeutic period, although this raises concerns regarding the likelihood of OHSS. Nonetheless, administering the drug at a low dose could mitigate this risk [17]. A study utilized the GnRH antagonist ganirelix to counteract the potential early rise in luteinizing hormone (LH) resulting from relatively high exposure to corifollitropin alfa during the initial days of stimulation [18]. Furthermore, in SAFA-FSH, both adjuvant GnRH agonists and antagonists can be employed to forestall an endogenous LH surge. Continuous research is imperative, yet it is envisaged that through these strategies, ART can be employed safely and conveniently, further streamlining ART treatment and rendering it more patient-friendly.

In addition to ART, oligoovulatory women with polycystic ovary syndrome (PCOS) undergoing ovulation induction with low-dose gonadotropins, the utilization of SAFA-FSH is anticipated to significantly improve patient convenience [19]. When a female fails to ovulate or conceive with letrozole or clomiphene, gonadotropin therapy may be recommended [20,21]. However, this treatment can lead to multiple pregnancies in patients with OHSS [22]. While low-dose or ultra-low-dose FSH might mitigate the risk, frequent injections can elevate stress levels and error rates [22-24]. The adoption of SAFA-FSH is expected to enhance adherence, minimize errors, and offer more consistent and beneficial effects due to its prolonged half-life. The increase in serum FSH levels between cycles surpasses the follicle recruitment threshold, thus affecting the number of follicles recruited [16]. Further investigation is necessary to establish the optimal dosage of SAFA-FSH.

In conclusion, SAFA-FSH holds promise as a long-acting hormone for treating female infertility. Its extended half-life could streamline treatment protocols, although additional research is required to refine dosing and address concerns, such as OHSS. Nonetheless, SAFA-FSH is poised to provide potential advantages in improving patient convenience and treatment outcomes in both ART and ovulation induction for women with PCOS.

CONFLICTS OF INTEREST

At the time of publication, Hyunjin Chi, Jeongsuk An, Kyungsun Lee, Jaekyu Han, Susan Chi, Moo Young Song, and Sang-Hoon Cha were employees of AprilBio Co. Ltd., Chuncheon, South Korea. The other authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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AUTHOR CONTRIBUTIONS

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