Original Research

Roles of kisspeptin in IVF/ICSI-treated infertile women and in human granulosa cells

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Impact statement

This study explored the effects of kisspeptin on different phases of IVF treatment including the beginning of gonadotropin stimulation (Phase I), around eight days after gonadotropin stimulation (Phase II), and the day of ovum pick-up (Phase III); and on steroidogenesis in human granulosa cells. In the human study, follicular fluid kisspeptin levels in successful subjects were higher than unsuccessful subjects; and were higher than its levels in serum at Phase III. Serum kisspeptin levels at Phase III were comparable with Phases I and II and were positively correlated with serum E2 in Phase II/III and outcomes of IVF treatment in successful subjects but were lower than Phase I in unsuccessful subjects. For the cell experiments, kisspeptin treatment enhanced *CYP19A1* (aromatase) mRNA expression and increased aromatase secretion. Taken together, kisspeptin enhanced aromatase expression and secretion in granulosa cells and might have a positive impact on IVF treatment.

Abstract

Kisspeptin, a crucial central regulator of reproduction, has been used as a trigger in *in vitro* fertilization (IVF) treatment. This study aimed to investigate the roles of kisspeptin in IVF treatment in infertile females ($n = 30$); and in steroidogenesis in human granulosa-like tumor cell line (KGN). In the human study, blood was collected at three time points including (1) the beginning of gonadotropin stimulation (Phase I), (2) around eight days after gonadotropin stimulation (Phase II), and (3) on the day of ovum pick-up (Phase III). Follicular fluid (FF) was collected at Phase III. Serum human chorionic gonadotropin (hCG) was measured 15 days after embryo transfer and fetal heart beats were determined around 42 days of menstrual cycle to classify the subjects into successful and unsuccessful groups. FF kisspeptin levels were higher in successful compared with unsuccessful subjects ($P < 0.01$). Kisspeptin levels were significantly higher in FF than in serum in successful subjects ($P < 0.05$) but were comparable in unsuccessful subjects. Serum kisspeptin was comparable among three phases in the successful group but its levels in Phase III were significantly lower compared with Phase I in the unsuccessful group ($P < 0.01$). Serum kisspeptin in Phase II/III had positive correlations with serum E2 in Phases II and III and the outcomes of IVF/intracytoplasmic sperm injection (ICSI) treatment including serum hCG levels. For the cell experiment ($n = 3$), kisspeptin treatment in the presence of FSH together with IGF-1 enhanced *CYP19A1* (aromatase) mRNA expression compared with control. FSH alone increased aromatase concentrations in the supernatant compared with control and kisspeptin at the dose of 10^{-2} mmol/L with FSH enhanced aromatase concentrations in the supernatant compared with FSH alone ($P < 0.001$ all). In conclusion, kisspeptin enhanced aromatase expression and secretion and was associated with positive outcomes of IVF/ICSI treatment. Further studies regarding supplementation of kisspeptin could reveal its beneficial effects on IVF/ICSI treatment.

Keywords: Infertility, IVF/ICSI treatment, kisspeptin, human granulosa cells

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Introduction

Infertility has been shown to be a global health problem.[1] The World Health Organization (WHO) estimated that 48.5 million couples are affected by infertility worldwide.[2] Particularly, in developing countries, percentage of infertility could reach to 30% occurring about one in every four couples.[1,3] *In vitro* fertilization (IVF) technique, the most useful treatment for infertility,[4] has been widely applied for optimizing the successful rate of pregnancy.

Kisspeptin, a hypothalamic neuropeptide, is encoded by *KISS1/Kiss1* gene.[5,6] The actions of kisspeptin are mediated via G-protein-coupled receptor 54 (GPR54) or KISS1/Kiss1 receptor (KISS1R/Kiss1r).[7,8] In the central regulation, kisspeptin has been recognized as the crucial and upstream

signal for activation of gonadotropin releasing hormone (GnRH) neurons to activate the hypothalamus–pituitary–gonadal (HPG) axis.[6] Furthermore, kisspeptin benefits oocyte maturation and potently stimulates GnRH/luteinizing hormone (LH) surge in the preovulatory phase to induce ovulation in non-human and human species.[9–15] Kisspeptin efficiently and safely triggered oocyte maturation and ovulation in IVF treatment.[16,17] In peripheral regulation, *KISS1/Kiss1* gene expression was detected in the liver, spleen, lung, heart, and pituitary gland in porcine as well as placenta and growing follicles in humans.[18,19] *KISS1R/Kiss1r* gene is expressed in pituitary gland, heart, and pancreas in murine as well as adipocytes, ovarian samples, and granulosa-lutein cells in humans.[19,20] Furthermore, KISS1R protein was localized in the theca cells of antral follicles and granulosa lutein cells in the corpus luteum in humans.[19] So, kisspeptin could probably exert a direct effect on cells in the gonad. Moreover, kisspeptin has been shown to be involved in steroidogenesis since kisspeptin enhanced progesterone secretion in chicken granulosa cells.[21]

Steroidogenesis refers to a process that cholesterol is taken to synthesize steroid hormones including progesterone and estrogen.[22] In theca cells, binding of LH to LH receptor stimulates the conversion of cholesterol to androstenedione which then is translocated to granulosa cells.[23] In granulosa cells, follicle stimulating hormone (FSH) binds to FSH receptor (FSHR) that activates aromatase leading to conversion of androstenedione to estrogen.[23] It is well known that the follicle growth is dependent on FSH[24] since FSH stimulates granulosa cell development[25] and follicle maturation.[26,27] Interestingly, FSH coupled with estradiol (E2) treatment significantly prevented follicular atresia;[28] increased the number of follicles (stages 1, 3–6) and the DNA content in isolated follicles (stages 1–6);[28] and rescued follicular development[29] compared with FSH treatment alone in female hypophysectomized mice[28] or *Kiss1* and *Kiss1r* knockout mice,[29] suggesting that E2 could exert a synergistic role on FSH to enhance follicular development and differentiation. In addition, E2 restored the survival of healthy follicles and the percentage of fast-growing follicles in monkeys after treatment with an inhibitor of steroid synthesis,[30] demonstrating that E2 seems to have some effects on primate follicular survival and growth. Furthermore, previous studies in humans reported that female patients with higher serum E2 on the day of ovulation induction had better outcomes of IVF/intracytoplasmic sperm injection (ICSI) treatment[31,32] including pregnancy rate,[31] suggesting that E2 might be associated with successful pregnancy. However, the roles of estrogen/kisspeptin in humans are not fully understood.

The aims of this study were to explore the roles of kisspeptin in IVF/ICSI treatment as well as investigate the direct effects of kisspeptin on *FSHR* and *CYP19A1* (aromatase) mRNA and aromatase protein in a medium. For the human study, we investigated blood levels of kisspeptin and other reproductive hormones in different stages of IVF/ICSI treatment and follicular fluid (FF) levels of kisspeptin on the day of ovum pick-up (OPU). We aimed to (1) compare blood levels of kisspeptin and reproductive hormones (anti-Müllerian hormone (AMH), LH, FSH, E2, and progesterone (P4)), FF levels of kisspeptin as well as outcomes of IVF/ICSI treatment between the successful and unsuccessful groups, (2) compare blood levels of kisspeptin and reproductive hormones (LH, FSH, E2, and P4) between different phases of IVF/ICSI treatment, (3) compare levels of kisspeptin between blood and FF, and (4) determine correlations of blood and FF levels of kisspeptin with reproductive hormones (AMH, LH, FSH, E2, and P4) and outcomes of IVF/ICSI treatment. For the cell study, we aimed to (1) investigate the effects of kisspeptin on *FSHR* and *CYP19A1* (aromatase) mRNA expressions in human granulosa cells and (2) investigate the effect of kisspeptin on aromatase concentrations in supernatant. Revelation of the roles of kisspeptin in IVF/ICSI treatment as well as its effects on steroidogenesis might lead to better understanding of hormonal interaction on reproductive regulation and/or increased strategies to improve IVF treatment.

Materials and methods

Participants

Infertile patients with indications for IVF/ICSI treatment were recruited at the Faculty of Medicine Siriraj Hospital between April 2018 and May 2019. The inclusion criteria were age 25–40 years, body mass index (BMI) of 18.5–24.9 kg/m^2, regular menstrual cycles (28–31 days), and intact ovaries bilaterally. Non-obese subjects ($BMI < 25\,kg/m^2$ according to the BMI classification for Asian population) were recruited because we would like to avoid the confounding effects of obesity on IVF treatment as a previous publication revealed that obesity could have negative impacts on oocyte maturation, ovulation, endometrial development, and implantation.[33] The exclusion criteria were history of endocrine diseases (i.e. hypo/hyperthyroidism, Cushing's syndrome, diabetes, and congenital adrenal hyperplasia), hormonal contraceptive use within two months (i.e. oral contraceptive pills and implantation), moderate/severe endometriosis, poor response to ovarian stimulation, and polycystic ovarian syndrome. A total of 30 subjects undergoing one complete cycle of IVF/ICSI treatment were recruited in the current study. Regarding the causes of infertility, 5 patients had endometriosis, 1 patient had myoma uteri, 2 patients had fallopian tube obstruction, 1 patient had hydrosalpinx, 2 patients had pelvic adhesion, 7 patients' partners had oligospermia, and 12 patients had unspecified causes. Among them, 10 out of 30 patients (33%) acquired successful clinical pregnancy and were classified into the successful group. On the other hand, 19 patients did not progress to clinical pregnancy and were classified into the unsuccessful group. Among them, 14 patients had embryo transfer (ET) but did not achieve clinical pregnancy while five patients did not acquire ET because of disqualified embryos. In addition, one patient suffered from ectopic pregnancy. The patient with ectopic pregnancy was only recruited for correlation analysis. The allocation of subjects of the IVF/ICSI treatment is shown in Figure 1.

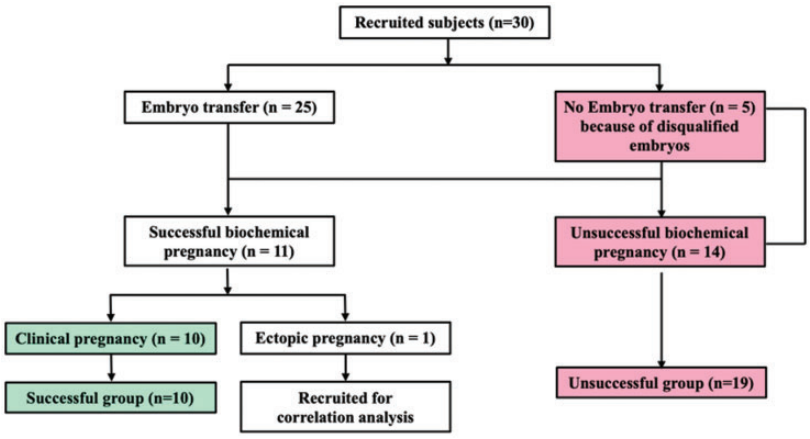


Figure 1. The allocation of subjects of the *in vitro* fertilization/intracytoplasmic sperm injection treatment. (A color version of this figure is available in the online journal.)

IVF/ICSI treatment protocol

The enrolled subjects underwent a complete cycle of IVF/ICSI treatment including ovarian stimulation, triggering for egg maturation, OPU, fertilization, and ET. There were three phases of IVF/ICSI treatment including the beginning of recombinant FSH (rFSH) stimulation (the early follicular phase or Phase I), around eight days after rFSH stimulation (the late follicular phase or Phase II), and on the day of OPU (the ovulatory phase or Phase III).

Ovarian stimulation protocol. On day 2 or 3 of the menstrual cycle (Phase I), blood was collected for measurement of LH, FSH, E2, P4, AMH, and kisspeptin; and transvaginal ultrasound (ALOKA SSD-3500SX) was performed for counting the number of antral follicles and checking the pathology of uterus and ovaries. Then, the subjects were subcutaneously injected with rFSH (Gonal-F, Merck Serono, Darmstadt, Germany) once daily around 11 days for follicle stimulation. On day 6 of rFSH stimulation, subcutaneous injection of GnRH antagonist (Cetrotide 0.25 mg, Merck Serono) was performed to prevent premature LH surge. Around eight days after rFSH stimulation (Phase II), a pelvic ultrasound was routinely carried out to evaluate the development of individual ovarian follicle. After ultrasound examination, blood samples were collected for measurement of LH, FSH, E2, P4, and kisspeptin (Phase II).

Triggering egg maturation. Around day 12 of menstrual cycle, a single subcutaneous bolus injection of human chorionic gonadotropin (hCG; Ovidrel, 250 mg/0.5 mL, Merck Serono) was administered to promote oocyte maturation.

OPU and follicular fluid collection. Around day 14 of menstrual cycle (Phase III), blood samples were collected for measurement of LH, FSH, E2, P4, and kisspeptin just prior to the transvaginal ultrasound-guided OPU, which was carried out 36 h following hCG administration under intravenous sedation. After oocyte removal, FF was collected and frozen for further analysis of kisspeptin. Furthermore, the number of collected oocytes and matured oocytes was counted. Then, after the procedure of OPU (the luteal phase), the subjects were supplemented with cyclogest (400 mg twice daily suppository) as P4 being the active ingredient and duphaston (10 mg orally 2 times daily) as dydrogesterone being the active ingredient until 10–12 weeks of gestation.

Fertilization and transferring of embryos. The retrieved oocytes (RO) were evaluated for their maturity. The matured oocytes (metaphase II, MII) were fertilized by ICSI. On the following day of fertilization, the embryologist determined and counted the number of 2 pronuclear (2PN) zygotes which were used for indicating successful fertilization. After three days of OPU, the embryos were evaluated by morphological assessment and 1–2 embryo(s) of suitable quality was/were transferred into the uterine cavity. However, patients with high risk of ovarian hyperstimulation syndrome (OHSS) or questioning about the abnormality of endometrium, the embryos were cryopreserved by vitrification.

Testing of biochemical pregnancy and clinical pregnancy. After 15 days of ET, blood hCG levels were measured for testing biochemical pregnancy. Around 42 days of menstrual cycle, successful clinical pregnancy was confirmed by ultrasound scan to observe the heart rate of fetus in patients who experienced successful biochemical pregnancy.

Hormonal assay methodology

Serum LH, FSH, E2, P4, and AMH levels were analyzed by the central laboratory of the Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand using electrochemiluminescence immunoassay (ECLIA; COBAS®, Trademark of Roche Diagnostics GmbH, Mannheim). According to the manufacturer's protocols, the ranges of detection were 0.01–23 ng/mL for AMH, 0.1–200 mIU/mL for LH, 0.1–200 mIU/mL for FSH, 5–3,000 pg/mL for E2, and 0.05–60 ng/mL for P4.

Serum and FF kisspeptin levels were measured by commercial enzyme immunoassays (EIA) kits (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). Based on the manufacturer's protocol, the range of kisspeptin

detection was 0–100 ng/mL. The intra-assay variation was 6.46%.

The human granulosa-like tumor cell line (KGN)

The human granulosa-like tumor cell line (KGN) was purchased from the RIKEN Bioresource center (Tsukuba, Japan). According to a previous study,[34] KGN cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (1:1) medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), and penicillin–streptomycin solution (Sigma-Aldrich, Saint Louis, MO, USA) in a 5% CO_2 at 37°C.

Chemical preparation and KGN cell treatment

All chemicals including purified kisspeptin-10 (Sigma), insulin-like growth factor 1 (IGF-1) (Sigma), and FSH (Sigma; and Abcam, Cambridge, UK) were dissolved in water which were then stored at −20°C until treatment. Serum free DMEM/F-12 medium was used for diluting chemicals according to different designed treatment concentrations. For kisspeptin, the stock was diluted to 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} mmol/L, respectively. In addition, the stocks of IGF-1 and FSH were diluted to 10^{-5} mmol/L according to the previous publication.[35] KGN cells were treated for 24 h with serum-free DMEM/F-12 medium as a control; 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and10^{-2} mmol/L kisspeptin; 10^{-5} mmol/L FSH; 10^{-5} mmol/L IGF-1; 10^{-5} mmol/L FSH together with 10^{-5} mmol/L IGF-1; 10^{-5} mmol/L FSH together with $10^{-6}/10^{-5}/10^{-4}/10^{-3}/10^{-2}$ mmol/L kisspeptin; and 10^{-5} mmol/L FSH together with 10^{-5} mmol/L IGF-1 and $10^{-6}/10^{-5}/10^{-4}/10^{-3}/10^{-2}$ mmol/L kisspeptin. During the treatment, the cell culture was performed in a humidified atmosphere with 5% CO_2 at 37°C. All experiments were done in triplicate, including control.

RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA of interested genes was extracted from KGN cells by the TRIzol® Reagent (InvitrogenTM, Carlsbad, CA, USA) according to the manufacture's protocol. RT-PCR was performed with the QPCR Green Master Mix LRox, 2X kit (Biotechrabbit, Berlin, Germany) for quantifying *FSHR* and *CYP19A1* (aromatase) genes. The hypoxanthine phosphoribosyltransferase 1 (*HRPT1*) gene was used as the reference gene for normalization as it appears to be the most stably expressed gene in granulosa cells.[36] The primers for the *HRPT1*, *FSHR*, and *CYP19A1* genes were designed by the authors and blasted to prove the specificity using published nucleotide sequences from PubMed database. The real-time PCR primer sequences are shown in Table 1.

The RT-PCR amplification was performed with Bio-rad Hercules CA94547 (Bio-Rad Laboratories, Inc. 2000, USA) according to the manufacturer's instructions. Then, the gene expressions of *FSHR* and *CYP19A1* were calculated using the $2^{-\Delta Ct}$ method.

Analysis of aromatase protein levels in supernatant

The supernatant was collected from each well of the culture plates and was kept in −80°C until analysis. Then, aromatase levels in supernatant were analyzed by a commercial ELISA kit (Elabscience Biotechnology Inc., Houston, TX, USA). The range of human aromatase detection was 0.16–10 ng/mL. The intra-assay variation was 6.03%.

Statistics

Statistical analysis was done by the Statistical Package for the Social Sciences (SPSS) version 18.0. Data were shown as mean ± SD. To test for a normal distribution of data, the Kolmogorov–Smirnov test was performed. Comparisons of two independent groups, between the successful and unsuccessful groups were performed by the independent Student's *t*-test. According to our findings showing that serum AMH levels in Phase I were significantly higher but basal serum FSH levels were significantly lower in the successful group compared with the unsuccessful group, so AMH in Phase I and basal FSH could be confounding factors for comparisons between the successful and unsuccessful groups. To overcome these limitations, the analysis of covariance (ANCOVA) was performed with AMH in Phase I or FSH in phase I or AMH and FSH in phase I adjustments to clearly reveal the roles of kisspeptin and other hormones on IVF/ICSI treatment. The comparison of kisspeptin levels between blood and FF was performed by the paired *t*-test. Comparisons of more than two independent groups, between different phases of IVF/ICSI treatment (Phases I, II, and III) as well as different treatments of KGN cells, were performed by the one-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) post hoc test. Comparisons of non-normal distributed data were assessed by non-parametric tests. Correlation coefficients were determined using the Pearson product–moment correlation coefficient method for normal distributed data or using the

Table 1. The real-time PCR primer sequences.

Gene	Nucleotide sequences of primer	Product size (bp)
FSHR	Forward-5′ AGG AAT GCC ATT GAA CTG AGG T 3′ Reverse-5′ CAG ATA TTG AAG GTT GGG AAG GT 3′	240
CYP19A1	Forward-5′ AGC AAG TCC TCA AGT ATG TTC CAC 3′ Reverse-5′ GTC CAC ATA GCC CGA TTC ATT 3′	267
HRPT1	Forward- 5′ CCT GGC GTC GTG ATT AGT GA 3′ Reverse-5′ CCA TCT CCT TCA TCA CAT CTC G 3′	156

FSHR: follicle stimulating hormone receptor; HRPT1: hypoxanthine phosphoribosyltransferase 1.

Spearman's Rank correlation coefficient method for non-normal distributed data. A *P*-value less than 0.05 was considered to be statistically significant.

Results

The comparisons of clinical parameters, hormonal levels in different phases, and the outcomes of IVF/ICSI treatment between the successful and unsuccessful groups

The comparisons of clinical parameters, hormonal levels in different phases, and the outcomes of IVF/ICSI treatment between the successful and unsuccessful groups are shown in Table 2. For clinical parameters, age, body weight, BMI, and the length of menstrual cycle were comparable between the successful and unsuccessful groups. The length of ovarian stimulation had a trend to be longer in the successful group compared with the unsuccessful group ($P = 0.067$; Table 2).

Hormonal levels were measured at Phases I, II, and III of IVF/ICSI treatment. For Phase I, serum kisspeptin, E2, and P4 were comparable between the successful and unsuccessful groups (Table 2). Serum AMH levels were significantly higher in the successful group compared with the unsuccessful group ($P < 0.05$) even after adjustment for FSH in Phase I ($P < 0.01$; Table 2). Serum LH levels were comparable between the successful and unsuccessful groups; however, it was significantly lower in the successful group compared with the unsuccessful group after adjustment for AMH and FSH in Phase I ($P < 0.05$; Table 2). Serum FSH levels were significantly lower in the successful group compared with the unsuccessful group even after adjustment for serum AMH in Phase I ($P < 0.05$ all; Table 2).

For Phase II, serum kisspeptin, LH, and P4 were comparable between the successful and unsuccessful groups. Serum E2 was also comparable between the successful and unsuccessful groups; however, after adjustment for AMH in Phase I or AMH and FSH in Phase I, it was significantly higher in the successful group compared with the

Table 2. The comparisons of clinical parameters, hormonal levels in different phases and the outcomes of IVF/ICSI treatment between the successful and unsuccessful groups.

				P value after adjusting for		
Parameters	**Success ($n = 10$)**	**Unsuccess ($n = 19$)**	***P* value**	**AMH (Phase I)**	**FSH (Phase I)**	**AMH & FSH (Phase I)**
Clinical parameters						
Age (year)	34.70 ± 3.09	35.58 ± 3.17	0.480			
Body weight (kg)	54.90 ± 5.94	51.90 ± 6.67	0.243			
BMI (kg/m^2)	21.30 ± 2.23	20.52 ± 1.73	0.306			
The length of menstrual cycle	30.60 ± 2.88	29.47 ± 3.53	0.394			
The length of ovarian stimulation (day)	10.67 ± 1.22	9.89 ± 0.88	0.067			
Phase I (the beginning of rFSH stimulation or the early follicular phase)						
Serum AMH (ng/mL)	4.46 ± 2.94	2.00 ± 0.99	0.028*		0.003**	
Serum kisspeptin (pg/mL)	527.93 ± 135.31	558.90 ± 80.94	0.445	0.435	0.552	0.588
Serum LH (mIU/mL)	4.74 ± 1.36	5.82 ± 1.95	0.128	0.106	0.106	0.032*
Serum FSH (mIU/mL)	6.47 ± 1.18	8.80 ± 2.53	0.011*	0.009*		
Serum E2 (pg/mL)	33.01 ± 11.21	44.38 ± 25.12	0.187	0.147	0.089	0.123
Serum P4 (ng/mL)	0.21 ± 0.19	0.21 ± 0.10	0.989	0.838	0.990	0.926
Phase II (around 8 days after rFSH stimulation or the late follicular phase)						
Serum kisspeptin (pg/mL)	595.80 ± 116.72	522.90 ± 111.15	0.144	0.199	0.261	0.351
Serum LH (mIU/mL)	3.64 ± 6.00	2.20 ± 1.32	0.303	0.216	0.435	0.381
Serum FSH (mIU/mL)	13.39 ± 4.17	17.98 ± 5.42	0.028*	0.015*	0.087	0.040*
Serum E2 (pg/mL)	1558.01 ± 957.37	1276.91 ± 654.44	0.365	<0.001***	0.154	0.001***
Serum P4 (ng/mL)	1.55 ± 3.67	0.43 ± 0.36	0.205	0.371	0.447	0.543
Phase III (the day of OPU or the ovulatory phase)						
Serum kisspeptin (pg/mL)	563.82 ± 157.17	484.21 ± 112.15	0.144	0.089	0.140	0.157
Serum LH (mIU/mL)	2.55 ± 1.85	2.18 ± 3.29	0.755	0.903	0.292	0.253
Serum FSH (mIU/mL)	10.05 ± 2.75	9.82 ± 3.59	0.872	0.723	0.262	0.348
Serum E2 (pg/mL)	1,337.82 ± 729.19	897.61 ± 491.58	0.069	<0.001***	0.010*	<0.001***
Serum P4 (ng/mL)	9.98 ± 7.01	9.65 ± 6.03	0.897	0.021*	0.025*	0.010*
Follicular fluid kisspeptin (pg/mL)	893.81 ± 60.93	543.52 ± 210.47	<0.001***	<0.001***	0.001**	<0.001***
The outcomes of IVF/ICSI treatment						
No. of RO/subject	18.20 ± 11.67	7.53 ± 4.36	0.019*	<0.001***	<0.001***	<0.001***
No. of MII/subject	13.10 ± 6.47	5.47 ± 3.49	<0.001***	<0.001***	<0.001***	<0.001***
No. of 2PN/subject	10.30 ± 5.44	4.11 ± 3.04	<0.001***	<0.001***	<0.001***	<0.001***
No. of transferred embryos/subject	2 ± 0.00	1.42 ± 0.90	0.012*	0.152	0.019*	0.020*
Serum hCG (mIU/mL)	1176.79 ± 1358.82	0.93 ± 2.36	0.004**	0.011*	0.014*	0.031*

Values are presented as mean ± SD.

BMI: body mass index; AMH: anti-Müllerian hormone; LH: luteinizing hormones; FSH: follicle stimulating hormone; E2: estradiol; P4: progesterone; FF: follicular fluid; No.: number; RO: retrieved oocytes; MII: matured oocytes; 2PN: 2 pronuclear; hCG: human chorionic gonadotropin.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared between the success and unsuccess groups.

unsuccessful group ($P < 0.001$ all; Table 2). Serum FSH levels were significantly lower in the successful group compared with the unsuccessful group even after adjustment for AMH in Phase I or AMH and FSH in Phase I ($P < 0.05$ all; Table 2).

For Phase III, serum kisspeptin, LH, and FSH levels were comparable between the successful and unsuccessful groups (Table 2). Serum E2 and P4 were comparable between the successful and unsuccessful groups; however, these hormones were significantly higher in the successful group compared with the unsuccessful group after adjustment for AMH in Phase I or FSH in Phase I or AMH and FSH in Phase I ($P < 0.05$ all; Table 2). Remarkably, FF kisspeptin levels were significantly higher in the successful group compared with the unsuccessful group ($P < 0.001$) even after adjustment for AMH in Phase I ($P < 0.001$) or FSH in Phase I ($P < 0.01$) or AMH and FSH in Phase I ($P < 0.001$; Table 2).

For the outcomes of IVF/ICSI treatment, the number of RO, MII, and 2PN per subject as well as serum hCG were significantly higher in the successful group compared with the unsuccessful group even after adjustment for AMH in Phase I or FSH in Phase I or AMH and FSH in Phase I ($P < 0.05$ all; Table 2). The number of transferred embryo(s) was significantly higher in the successful group compared with the unsuccessful group even after adjustment for FSH in Phase I or AMH and FSH in Phase I ($P < 0.05$ all; Table 2).

The levels of hormones in different phases of IVF/ICSI treatment as well as the comparison of kisspeptin levels between serum and follicular fluid in total, successful, and unsuccessful subjects

The levels of hormones in different phases of IVF/ICSI treatment as well as the comparison of kisspeptin levels between serum and FF in total, successful, and unsuccessful subjects are shown in Figure 2. Serum kisspeptin levels in Phase III were significantly lower compared with Phase I ($P < 0.01$) and Phase II ($P < 0.05$) in total subjects and compared with Phase I in unsuccessful subjects ($P < 0.01$; Figure 2(a)). However, serum kisspeptin levels were comparable among different phases of IVF/ICSI treatment in successful subjects (Figure 2(a)).

Serum LH levels in Phase I were significantly higher than Phase II and Phase III in total and unsuccessful subjects ($P < 0.001$ all) and higher than Phase III in successful subjects ($P < 0.001$; Figure 2(b)). Serum FSH levels in Phase II were significantly higher compared with Phase I and Phase III in total and unsuccessful subjects ($P < 0.001$ all) and than Phase I in successful subjects ($P < 0.01$).

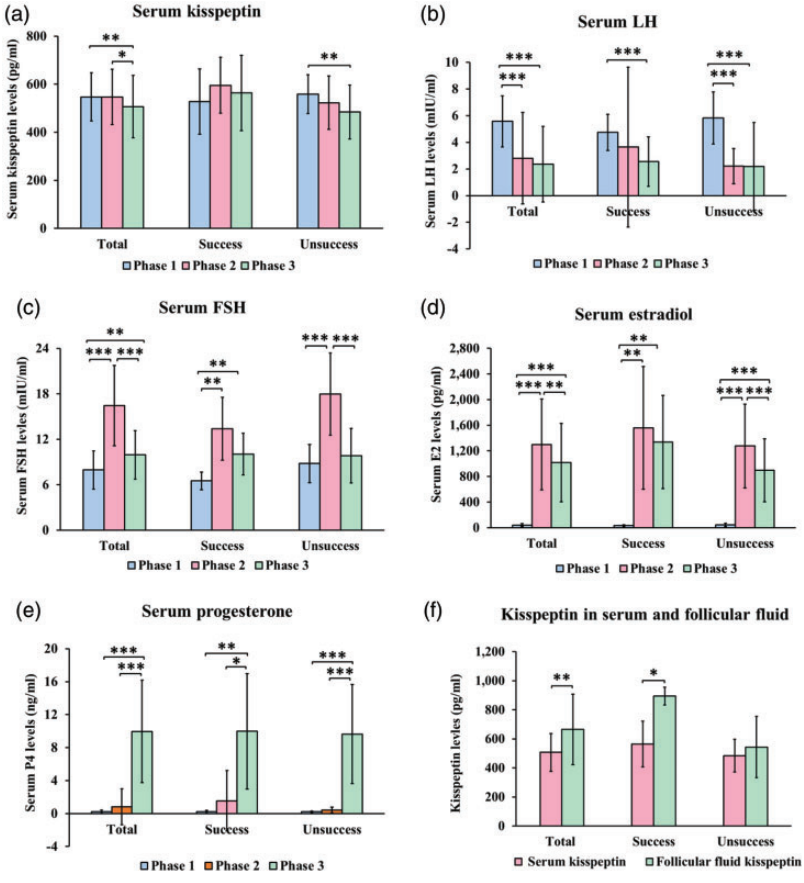


Figure 2. The levels of hormones in different phases of *in vitro* fertilization/intracytoplasmic sperm injection treatment as well as the comparison of kisspeptin levels between serum and follicular fluid in total, successful, and unsuccessful subjects. (a) serum kisspeptin levels; (b) serum LH levels; (c) serum FSH levels; (d) serum estradiol levels; (e) serum progesterone levels; (f) kisspeptin levels in serum and follicular fluid in Phase III; LH: luteinizing hormone; FSH: follicle stimulating hormone; Phase I: the beginning of rFSH stimulation (the early follicular phase); Phase II: around eight days after rFSH stimulation (the late follicular phase); Phase III: on the day of OPU (the ovulatory phase). Values are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Furthermore, in total subjects, serum FSH levels in Phase III were significantly higher compared with Phase I ($P < 0.01$; Figure 2(c)). Serum E2 levels in Phase II were significantly higher compared with Phase I and Phase III ($P < 0.01$ all) and in Phase III were significantly higher compared with Phase I ($P < 0.001$ all) in total and unsuccessful subjects. In successful subjects, serum E2 levels in Phase I were significantly lower compared with Phase II and Phase III ($P < 0.01$ all; Figure 2(d)). Serum P4 levels in Phase III were significantly higher than Phase I and Phase II in total ($P < 0.001$ all), successful ($P < 0.05$ all), and unsuccessful subjects ($P < 0.001$ all; Figure 2(e)).

FF kisspeptin levels were significantly higher than its levels in serum in total ($P < 0.01$) and successful ($P < 0.05$) subjects but were comparable in unsuccessful subjects (Figure 2(f)).

Correlations of serum levels of AMH, kisspeptin, LH, and FSH with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase I

Correlations of serum levels of AMH, kisspeptin, LH, and FSH with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase I are shown in Table 3.

Serum AMH levels in Phase I had significant positive correlations with serum E2 in Phases II and Phase III, serum P4 in Phases II and Phase III, and the number of RO, MII, and 2PN in all subjects ($P < 0.05$ all); with serum LH in Phase I, serum E2 in Phase II and Phase III, and the number of RO, MII, and 2PN in successful subjects ($P < 0.05$ all); and with serum E2 in Phase II and Phase III, serum P4 in Phase II and Phase III, and the number of RO, MII, and 2PN in unsuccessful subjects ($P < 0.05$ all); but had significant negative correlations with serum FSH in Phase I and Phase II in all and unsuccessful subjects ($P < 0.05$ all; Table 3). Serum kisspeptin levels in Phase I had significant negative correlations with serum LH in Phase II and serum kisspeptin in Phase III in all subjects ($P < 0.05$ all) and with serum LH in Phase II in successful subjects ($P < 0.05$; Table 3). Serum LH levels in Phase I had significant positive correlations with serum LH in Phase III in all subjects ($P < 0.001$); with serum E2 in Phase II, serum P4 in Phase III, the number of RO and 2PN, and serum hCG in successful subjects ($P < 0.05$ all); and with serum LH in Phase III in unsuccessful subjects ($P < 0.05$); but had a significant negative correlation with serum E2 in Phase I in successful subjects ($P < 0.01$; Table 3). Serum FSH levels in Phase I had

Table 3. Correlations of serum levels of AMH, kisspeptin, LH, and FSH with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase I.

		All subjects ($n = 30$)		Success ($n = 10$)		Unsuccess ($n = 19$)	
Phase I	**Parameters**	***R***	***P***	***R***	***P***	***R***	***P***
Serum AMH	LH (Phase I)	0.068	0.728	0.742	0.014*	−0.058	0.818
	FSH (Phase I)	−0.702	<0.001***	−0.345	0.328	−0.644	0.004**
	FSH (Phase II)	−0.616	<0.001***	−0.406	0.244	−0.590	0.013*
	E2 (Phase II)	0.674	<0.001***	0.864	0.001**	0.494	0.044*
	E2 (Phase III)	0.756	<0.001***	0.900	0.001**	0.721	0.001**
	P4 (Phase II)	0.394	0.038*	0.358	0.310	0.712	0.001**
	P4 (Phase III)	0.463	0.013*	0.267	0.488	0.772	<0.001***
	No. of RO	0.890	<0.001***	0.867	0.001**	0.768	<0.001***
	No. of MII	0.755	<0.001***	0.794	0.006**	0.708	0.001**
	No. of 2PN	0.818	<0.001***	0.835	0.003**	0.627	0.005**
Serum kisspeptin	LH (Phase II)	−0.545	0.002**	−0.753	0.012*	0.138	0.586
	Kiss (Phase III)	−0.402	0.031*	−0.150	0.700	−0.212	0.384
Serum LH	LH (Phase III)	0.478	0.009**	0.583	0.099	0.527	0.021*
	E2 (Phase I)	−0.119	0.532	−0.815	0.004**	−0.085	0.731
	E2 (Phase II)	0.189	0.326	0.876	0.001**	0.068	0.787
	P4 (Phase III)	0.080	0.680	0.676	0.046*	−0.198	0.416
	No. of RO	0.122	0.522	0.651	0.042*	0.195	0.424
	No. of 2PN	0.135	0.476	0.699	0.024*	0.332	0.166
	Serum hCG	−0.054	0.796	0.648	0.043*	0.092	0.755
Serum FSH	LH (Phase II)	−0.254	0.184	−0.636	0.048*	0.052	0.837
	E2 (Phase II)	−0.410	0.027*	−0.632	0.050	−0.368	0.133
	E2 (Phase III)	−0.509	0.005**	−0.517	0.154	−0.495	0.031*
	P4 (Phase II)	−0.074	0.703	0.200	0.580	−0.549	0.018*
	P4 (Phase III)	−0.572	0.001**	−0.150	0.700	−0.658	0.002**
	No. of RO	−0.791	<0.001***	−0.522	0.098	−0.665	0.002**
	No. of MII	−0.600	<0.001***	−0.656	0.039*	−0.509	0.026*
	No. of 2PN	−0.680	<0.001***	−0.651	0.042*	−0.462	0.046*

AMH: anti-Müllerian hormone; LH: luteinizing hormones; FSH: follicle stimulating hormone; E2: estradiol; P4: progesterone; Kiss: kisspeptin; No.: number; RO: retrieved oocytes; MII: matured oocytes; 2PN: 2 pronuclear; hCG: human chorionic gonadotropin; Phase I: the beginning of gonadotropin stimulation (the early follicular phase); Phase II: around 8 days of rFSH stimulation (the late follicular phase); Phase III: on the day of OPU (the ovulatory phase).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

significant negative correlations with serum E2 in Phase II and Phase III, serum P4 in Phase III, and the number of RO, MII, and 2PN in all subjects ($P < 0.05$ all); with serum LH in Phase II and the number of MII and 2PN in successful subjects ($P < 0.05$ all); and with serum E2 in Phase III, serum P4 in Phases II and III, and the number of RO, MII, and 2PN in unsuccessful subjects ($P < 0.05$ all; Table 3).

Correlations of serum levels of kisspeptin, LH, and E2 with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase II

Correlations of serum levels of kisspeptin, LH, and E2 with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase II are shown in Table 4.

Serum kisspeptin levels in Phase II had significant positive correlations with serum LH in Phase II, serum kisspeptin in Phase III, the number of RO, 2PN, and serum hCG in all subjects ($P < 0.05$ all); with serum LH in Phase I and Phase II, serum kisspeptin in Phase III, the number of RO, and serum hCG in successful subjects ($P < 0.05$ all); and with serum P4 in Phase II in unsuccessful subjects ($P < 0.05$); but had a significant negative correlation with serum E2 in Phase I in successful subjects ($P < 0.05$; Table 4). Serum LH levels in Phase II had significant positive correlations with serum E2 in Phase II and Phase III and the number of RO, MII, and 2PN in all subjects ($P < 0.05$ all); and with serum E2 in Phase II and Phase III and the number of RO, MII, and 2PN in successful subjects ($P < 0.05$ all; Table 4). Serum E2 levels in Phase II had significant positive correlations with serum E2 in Phase III, serum P4 in Phase II and Phase III, and the number of RO, MII, and 2PN in all subjects ($P < 0.05$ all); with the number of RO, MII, and 2PN in successful subjects ($P < 0.05$ all); and with serum E2 in Phase III, serum P4 in Phase II and Phase III in unsuccessful subjects ($P < 0.01$ all; Table 4).

Correlations of serum levels of kisspeptin and E2 as well as follicular fluid levels of kisspeptin with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase III

Correlations of serum levels of kisspeptin and E2 as well as FF levels of kisspeptin with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase III are shown in Table 5.

Serum kisspeptin in Phase III had significant positive correlations with serum LH in Phase II, serum E2 in Phase III, the number of RO, MII, 2PN, and serum hCG in all subjects ($P < 0.05$ all); with serum LH in Phase II, serum E2 in Phase II and Phase III, the number of MII, 2PN, and serum hCG in successful subjects ($P < 0.05$ all); and with serum P4 in Phase I in unsuccessful subjects ($P < 0.01$; Table 5). Serum E2 levels in Phase III had significant positive correlations with the number of RO, MII, and 2PN in all and successful subjects ($P < 0.001$ all); and with serum P4 in Phase II and Phase III in unsuccessful subjects ($P < 0.001$; Table 5). FF kisspeptin levels in Phase III had significant positive correlations with serum AMH in Phase I and the number of MII and 2PN in all and unsuccessful subjects ($P < 0.05$ all) and with serum AMH in Phase I and serum E2 in Phase II in successful subjects ($P < 0.05$ all); but had

Table 4. Correlations of serum levels of kisspeptin, LH, and E2 with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase II.

		All subjects ($n = 30$)		Success ($n = 10$)		Unsuccess ($n = 19$)	
Phase II	**Parameters**	***R***	***P***	***R***	***P***	***R***	***P***
Serum kisspeptin	LH (Phase I)	0.222	0.248	0.787	0.007**	0.310	0.210
	LH (Phase II)	0.455	0.013*	0.806	0.005**	−0.057	0.791
	E2 (Phase I)	−0.181	0.346	−0.669	0.035*	−0.027	0.915
	P4 (Phase II)	−0.076	0.695	−0.374	0.288	0.537	0.022*
	Kiss (Phase III)	0.682	<0.001***	0.867	0.002**	0.442	0.066
	No. of RO	0.465	0.011*	0.643	0.045*	0.099	0.695
	No. of 2PN	0.445	0.016*	0.566	0.088	0.149	0.556
	hCG	0.438	0.032*	0.636	0.048*	0.197	0.518
Serum LH	E2 (Phase II)	0.379	0.042*	0.784	0.007**	0.080	0.753
	E2 (Phase III)	0.486	0.009**	0.739	0.023*	0.001	0.998
	No. of RO	0.707	<0.001***	0.882	0.001**	−0.081	0.750
	No. of MII	0.587	0.001***	0.745	0.013*	−0.056	0.826
	No. of 2PN	0.571	0.001**	0.793	0.006**	−0.055	0.828
Serum E2	E2 (Phase III)	0.761	<0.001***	0.617	0.077	0.890	<0.001***
	P4 (Phase II)	0.438	0.018*	0.176	0.626	0.684	0.002**
	P4 (Phase III)	0.603	0.001**	0.367	0.332	0.663	0.003**
	No. of RO	0.570	0.001**	0.772	0.009**	0.430	0.075
	No. of MII	0.507	0.005**	0.729	0.017*	0.301	0.205
	No. of 2PN	0.526	0.003**	0.786	0.007**	0.240	0.338

LH: luteinizing hormones; E2: estradiol; P4: progesterone; Kiss: kisspeptin; No.: number; RO: retrieved oocytes; MII: matured oocytes; 2PN: 2 pronuclear; hCG: human chorionic gonadotropin; Phase I: the beginning of rFSH stimulation (the early follicular phase); Phase II: around eight days after rFSH stimulation (the late follicular phase); Phase III: on the day of OPU (the ovulatory phase).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 5. Correlations of serum levels of kisspeptin and E2 as well as follicular fluid levels of kisspeptin with serum hormonal levels and outcomes of IVF/ICSI treatment in Phase III.

		All subjects ($n = 30$)		Success ($n = 10$)		Unsuccess ($n = 19$)	
Phase III	**Parameters**	***R***	***P***	***R***	***P***	***R***	***P***
Serum kisspeptin	LH (Phase II)	0.594	0.001**	0.933	<0.001***	−0.032	0.899
	E2 (Phase II)	0.291	0.133	0.708	0.033**	−0.104	0.682
	E2 (Phase III)	0.380	0.042*	0.700	0.036*	−0.075	0.761
	P4 (Phase I)	0.112	0.564	−0.168	0.666	0.645	0.003*
	No. of RO	0.601	0.001**	0.667	0.050	0.102	0.677
	No. of MII	0.547	0.002**	0.683	0.042*	0.109	0.656
	No. of 2PN	0.562	0.002**	0.728	0.026*	0.142	0.563
	hCG	0.431	0.035*	0.717	0.030*	0.108	0.714
Serum E2	P4 (Phase II)	0.205	0.297	0.133	0.732	0.816	<0.001***
	P4 (Phase III)	0.359	0.056	0.200	0.606	0.750	<0.001***
	No. of RO	0.731	<0.001***	0.817	0.007**	0.526	0.021
	No. of MII	0.689	<0.001***	0.816	0.008**	0.366	0.123
	No. of 2PN	0.682	<0.001***	0.895	0.001**	0.294	0.221
Follicular fluid kisspeptin	AMH	0.633	0.001**	0.802	0.017*	0.538	0.032*
	E2 (Phase I)	−0.503	0.009**	0.024	0.955	−0.494	0.044*
	E2 (Phase II)	0.122	0.563	0.790	0.020*	0.010	0.972
	No. of MII	0.640	<0.001***	0.395	0.333	0.549	0.023*
	No. of 2PN	0.645	<0.001***	0.515	0.192	0.598	0.011*

LH: luteinizing hormones; E2: estradiol; P4: progesterone; No.: number; RO: retrieved oocyte; MII: matured oocyte; 2PN: 2 pronuclear; hCG: human chorionic gonadotropin; Phase I: the beginning of gonadotropin stimulation (the early follicular phase); Phase II: around eight days after rFSH stimulation (the late follicular phase); Phase III: on the day of OPU (the ovulatory phase).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

significant negative correlations with serum E2 in Phase I in all and unsuccessful subjects ($P < 0.05$ all; Table 5).

The summary of results of IVF/ICSI treatment

The summary of results of IVF/ICSI treatment is shown in Figure 3 including the hormonal changes across different phases of IVF/ICSI treatment (Figure 3(a)) and comparisons of factors between the successful and unsuccessful groups as well as correlations of factors across different phases of IVF/ICSI treatment in the successful and unsuccessful groups (Figure 3(b)).

The effects of kisspeptin on KGN cell treatment

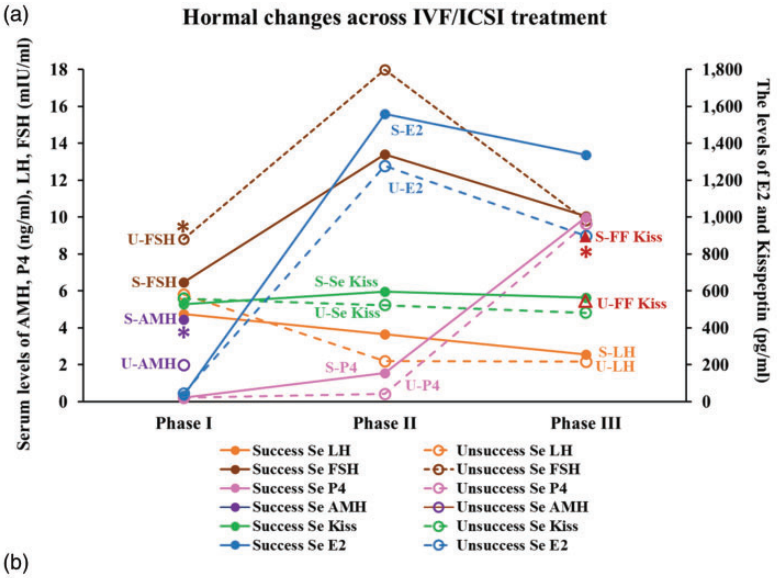
The effects of kisspeptin on KGN cell treatment are shown in Figure 4 consisting of Figure 4(a) for *FSHR* mRNA expression, Figure 4(b) for *CYP19A1* (aromatase) mRNA expression, and Figure 4(c) for aromatase protein concentrations in supernatant.

For *FSHR* mRNA expression, treatments of 10^{-5} mmol/L FSH, 10^{-5} mmol/L IGF-1; different doses (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} mmol/L) of kisspeptin; FSH together with different doses of kisspeptin; 10^{-5} mmol/L FSH together with 10^{-5} mmol/L IGF-1; and FSH together with IGF-1 and different doses of kisspeptin had no effect compared with control (Figure 4(a)).

For *CYP19A1* (aromatase) mRNA expression, treatments of 10^{-5} mmol/L FSH; 10^{-5} mmol/L IGF-1; all doses of kisspeptin; FSH together with all doses of kisspeptin; 10^{-8} M FSH together with 10^{-5} mmol/L IGF-1; and FSH together with IGF-1 and 10^{-6} and 10^{-5} mmol/L kisspeptin had no effect compared with control (Figure 4(b)). Interestingly, kisspeptin at the doses of 10^{-4}, 10^{-3}, and 10^{-2} mmol/L in the presence of 10^{-5} mmol/L FSH together with 10^{-5} mmol/L IGF-1 significantly increased *CYP19A1* (aromatase) mRNA expression compared with control ($P < 0.05$; Figure 4(b)).

For aromatase protein concentrations in the supernatant, treatment of 10^{-8} M FSH significantly increased aromatase concentrations in the supernatant (5.05 ± 0.94 ng/mL) compared with control (0.13 ng/mL; $P < 0.001$; Figure 4(c)). Treatments of 10^{-8} M IGF-1 and all doses of kisspeptin had no effect on aromatase concentrations in the supernatant compared with control (Figure 4(c)). FSH together with 1, 10, 100, and 1000 nM kisspeptin significantly increased aromatase concentrations in the supernatant (5.69 ± 1.04 ng/mL, 4.66 ± 0.22 ng/mL, 5.09 ± 1.45 ng/mL, and 3.08 ± 0.53 ng/mL, respectively, $P < 0.001$ all) compared with control but had comparable effect with FSH treatment alone (Figure 4(c)). Interestingly, FSH together with 10,000 nM kisspeptin significantly increased aromatase concentrations in the supernatant (10.26 ± 1.63 ng/mL) compared with control as well as FSH treatment ($P < 0.001$ all; Figure 4(c)). Concentrations of aromatase in the supernatant were significantly increased after treatment with 10^{-8} M FSH and 10^{-8} M IGF-1 (4.72 ± 0.63 ng/mL), 10^{-8} M FSH together with 10^{-8} M IGF-1 and 1, 10, 100, and 1000 nM kisspeptin (4.26 ± 0.04 ng/mL, 3.77 ± 0.63 ng/mL, 5.19 ± 0.31 ng/mL, and 4.04 ± 0.21 ng/mL, respectively; $P < 0.001$ all) compared with control but had comparable effect with FSH treatment alone (Figure 4(c)). Remarkably, treatment of 10^{-8} M FSH together with 10^{-8} M IGF-1 and 10,000 nM kisspeptin statistically increased aromatase concentrations in the supernatant (8.41 ± 0.57 ng/mL) compared with control ($P < 0.001$) as well as 10^{-8} M FSH and 10^{-5} mmol/L IGF-1 treatment ($P < 0.01$; Figure 4(c)).

(a)



(b)

Timeline of IVF/ICSI treatment	Successful compared to unsuccessful group	Correlations		
		Factors	Successful group	Unsuccessful group
Phase I	↑AMH, ↓FSH	Se-Kiss	⊖ LH (Phase II)	⊕ Kiss (Phase III)
		AMH	⊕ LH (Phase I), E2 (Phase II & III), No. of RO, MII, 2PN	⊕ P4 (Phase II), E2 (Phase II), E2 & P4 (Phase III), No. of RO, MII, 2PN ⊖ FSH and E2 (Phase I), FSH (Phase III)
		LH	⊕ E2 (Phase II), P4 (Phase III), No. of RO, 2PN, Serum hCG ⊖ E2 (Phase I)	⊕ LH (Phase III)
		FSH	⊖ LH, E2 (Phase II), No. of RO, 2PN	⊖ P4 (Phase II & III), E2 (Phase III), No. of RO, MII, 2PN
Phase II	↓FSH	Se-Kiss	⊕ LH (Phase I & II), Kiss (Phase III), No. of RO, Serum hCG ⊖ E2 (Phase I)	⊕ P4 (Phase II)
		LH	⊕ E2 (Phase II & III), No. of RO, MII, 2PN	
		E2	⊕ No. of Ro, MII, 2PN	⊕ P4 (Phase II & III), E2 (Phase III) ⊖ P4 (Phase I)
Phase III	↑E2 (P=0.069) ↑FF-Kiss ↑No. of RO ↑No. of MII	Se-Kiss	⊕ LH (Phase II), E2 (Phase II & III), No. of RO, MII, 2PN, Serum hCG	⊕ P4 (Phase I)
		E2	⊕ No. of RO, MII, 2PN	⊕ P4 (Phase II & III), No. of RO
		FF-Kiss	⊕ AMH (Phase I), E2 (Phase II)	⊕ AMH (Phase I), No. of MII, 2PN ⊖ E2 (Phase I)
Fertilization	↑No. of 2PN			
Embryo Transfer	↑No. of embryo			
Bio. pregnancy	↑Se hCG			
Cli. pregnancy	↑Heart beat			

Figure 3. The summary of results of *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment. (a) The hormonal changes across different phases of IVF/ICSI treatment; (b) The comparisons of factors between the successful and unsuccessful groups as well as the correlations of factors in different phases of IVF/ICSI treatment in the successful and unsuccessful groups. Filled cycles/triangles represent the mean of the successful group while empty circles/triangles represent the mean of the unsuccessful group. Solid lines represent the successful group while dash lines represent the unsuccessful group; S: success; U: unsuccess; Se: serum; FF: follicular fluid; Bio.: biochemical; Cli.: clinical; Kiss: kisspeptin; AMH: anti-Müllerian hormone; LH: luteinizing hormones; FSH: follicle stimulating hormone; E2: estradiol; P4: progesterone; No.: number; RO: retrieved oocytes; MII: matured oocytes; 2PN: 2 pronuclear; hCG: human chorionic gonadotropin; Phase I: the beginning of rFSH stimulation (the early follicular phase); Phase II: around eight days after rFSH stimulation (the late follicular phase); Phase III: on the day of OPU (the ovulatory phase); Success: the successful group; Unsuccess: the unsuccessful group ↑: increased, ↓: decreased, ⊕: positive correlation, ⊖: negative correlation. *$P < 0.05$.

Discussion

Kisspeptin has been well-known to exert central effect on hypothalamus to enhance the activity of GnRH neurons;[6,37,38] however, the roles of kisspeptin in various phases of IVF treatment have not been elucidated. We therefore investigate the roles of kisspeptin in different phases of IVF/ICSI treatment. Furthermore, the roles of kisspeptin on steroidogenesis were studied to reveal its peripheral reproductive functions. To the best of our knowledge, this is the first study to compare kisspeptin and reproductive hormones between the successful and unsuccessful groups across three phases of IVF/ICSI treatment; compare kisspeptin and reproductive hormones among three phases of IVF/ICSI treatment with subgroup analysis of subjects into the successful and unsuccessful groups; and reveal correlations of blood and FF levels of kisspeptin with serum hormonal levels and outcomes in different stages of IVF/ICSI treatment with subgroup analysis of subjects into the successful and unsuccessful groups.

In the present study, clinical parameters of infertile patients including age, body weight, BMI, and the length of menstrual cycle were comparable between successful and unsuccessful IVF/ICSI-treated subjects. Remarkably, FF kisspeptin levels in Phase III were significantly higher in the successful group compared with the unsuccessful group. Furthermore, FF kisspeptin levels in Phase III were significantly higher than its levels in serum in Phase III in total and successful subjects but were comparable in unsuccessful subjects. Our results in total and successful subjects were similar with a previous study reporting that FF kisspeptin levels on the OPU day were significantly

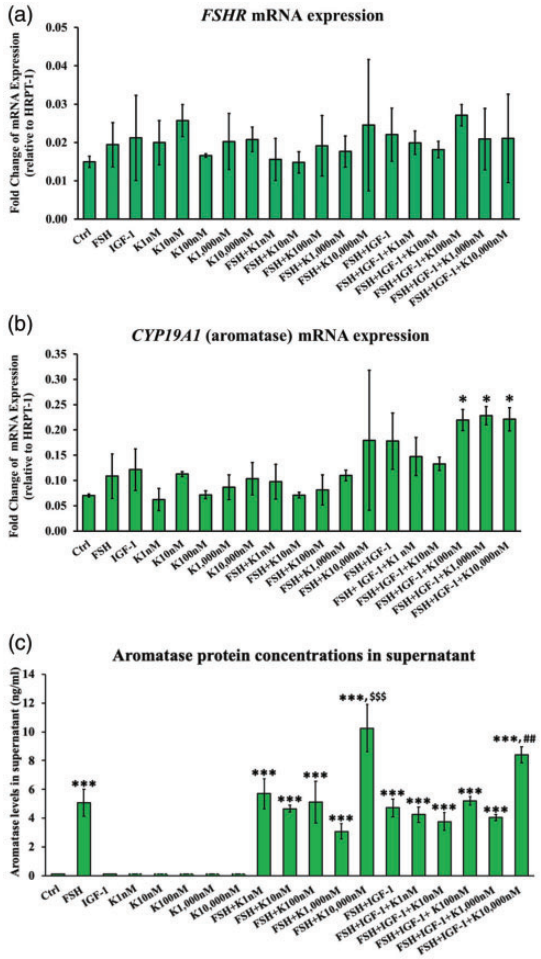


Figure 4. The effects of kisspeptin on KGN cell treatment ($n = 3$). (a) *FSHR* mRNA expression; (b) *CYP19A1* (aromatase) mRNA expression; (c) aromatase protein concentrations in supernatant. Data are presented as mean ± SD. Ctrl: control; K: kisspeptin; FSH: follicle stimulating hormone; IGF-1: insulin-like growth factor-1; FSHR: FSH receptor. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control, $^{\$\$\$}P < 0.001$ compared with FSH treatment, $^{\#\#}P < 0.01$ compared with FSH + IGF-1 treatment. (A color version of this figure is available in the online journal.)

higher than its levels in plasma.[39] Additionally, FF kisspeptin levels in Phase III were positively correlated with the number of MII and 2PN in total and unsuccessful subjects. These results together indicate that higher levels of kisspeptin in FF might be contributed to the success of IVF/ICSI treatment.

Serum kisspeptin levels in all three phases showed no significant differences between successful and unsuccessful subjects. However, the levels of kisspeptin in Phase III were significantly decreased compared with Phase I in total and unsuccessful subjects but were comparable in successful subjects. Serum kisspeptin levels in Phase III showed positive correlations with the outcomes of IVF/ICSI treatment including the number of RO, MII, and 2PN and serum hCG. Taken together, these results suggest that higher levels of FF kisspeptin in Phase III in successful subjects could probably result in maintenance of kisspeptin levels in Phase III which was associated with the positive outcomes of IVF/ICSI treatment. However, in the unsuccessful group, reduced FF kisspeptin levels in Phase III might be related to the decrease in its levels in serum in Phase III. The results regarding decreased serum kisspeptin levels in Phase III in total and unsuccessful subjects were inconsistent with a previous study from Taniguchi *et al.* in which the highest levels of plasma kisspeptin were observed on the day of OPU (Phase III) compared with the beginning of ovarian stimulation (Phase I).[39] The previous study recruited 30 subjects with 9 successful and 21 unsuccessful outcomes. Among these patients, serum samples were collected from only 14 patients with no details of the number of successful/unsuccessful subjects. We speculated that the inconsistent results might be from different proportion of successful and unsuccessful patients leading to contradictory kisspeptin levels in Phase III of IVF/ICSI treatment.

Remarkably, serum kisspeptin in Phase III had positive correlations with serum E2 in Phase II and Phase III and FF kisspeptin levels in Phase III had positive correlations with serum E2 in Phase II in the successful group. In the KGN cells study, we found that kisspeptin-10 together with FSH and IGF-1 enhanced *CYP19A1* (aromatase) mRNA expression and aromatase secretion in the supernatant suggesting that kisspeptin might increase the aromatase action, which might lead to increased E2 synthesis. Our results were in accordance with a previous study in IVF-treated patients showing that *CYP19A1* gene expression in granulosa cells was significantly higher in the kisspeptin-54 triggering group than the hCG or GnRH agonist group.[40]

Serum E2 in Phase III had a trend to be higher in the successful group than the unsuccessful group ($P = 0.069$). After adjustment for AMH in Phase I or FSH in Phase I or AMH and FSH in Phase I, the E2 levels were significantly higher in successful subjects than unsuccessful subjects. In addition, serum E2 levels in Phase II were significantly higher in the successful group compared with the unsuccessful group after adjustment for AMH in Phase I or AMH and FSH in Phase I. Serum E2 levels in Phase III were comparable with Phase II in the successful group but were lower than Phase II in the unsuccessful group. Serum E2 levels in Phase II and Phase III were positively correlated with the numbers of RO, MII, and 2PN only in the successful group. These results suggested that serum E2 in Phase II and Phase III might be positively associated with the outcomes of IVF/ICSI treatment. Our results were in agreement with previous studies[31,32] revealing that patients with higher serum E2 levels (more than 3500 pg/mL[31] or 4000 pg/mL[32]) on the day of hCG administration had better outcomes of IVF/ICSI treatment (higher numbers of RO/obtained embryos/transferred embryos/or pregnancy rate) compared with their counterparts. On the other hand, a meta-analysis reported that oral administration of E2 during the luteal phase failed to improve the outcomes of IVF or ICSI treatment including clinical pregnancy rate (CPR) per patient, CPR per ET, implantation rate, ongoing pregnancy rate per patient, clinical abortion rate, and ectopic pregnancy rate.[41] Although E2 supplement during luteal phase did not show a beneficial effect on IVF treatment, we believe that high E2 levels in the follicular phase (Phase II and Phase III of IVF treatment) might have a positive effect. E2 has been used to estimate the maturation of growing follicles in humans,[42] suggestive of the higher E2 levels,

the more mature follicles. Furthermore, previous studies in animals reported that E2 was important for development of secondary follicles in mice,[43] could exert a synergistic role on FSH to enhance follicular development and differentiation in mice,[28,29] and seems to have some effects on follicular survival and growth in monkeys.[30] Thus, we hypothesized that E2 in the follicular phase in humans might exert a positive impact on follicular development/maturation and outcomes of IVF treatment.

We proposed that granulosa cells of successful subjects could secrete higher levels of kisspeptin into FF leading to enhanced aromatase expression and secretion probably leading to increased E2 levels. Furthermore, we speculated that higher FF levels of kisspeptin in Phase III in the successful group might lead to maintenance of serum kisspeptin and E2 levels in Phase III compared with Phase II while lower FF levels of kisspeptin in Phase III in the unsuccessful group probably results in lower serum kisspeptin and E2 levels in Phase III compared with Phase II. The sustained high levels of kisspeptin and E2 in Phase III might be important for the successful outcomes of IVF/ICSI treatment as we found positive correlations of serum kisspeptin/E2 in Phase III with the outcomes of IVF/ICSI treatment as well as serum hCG. Our hypothesis is supported by a previous study showing that the addition of kisspeptin-54 after 10 h of first kisspeptin-54 trigger showed higher oocyte yield, implantation rate, and the live birth rate compared with a single kisspeptin-54 trigger.[44] On the other hand, in the unsuccessful group, we postulated that there might be a defect in kisspeptin synthesis/secretion in/from granulosa cells leading to lower serum kisspeptin levels in Phase III.

Serum LH levels in Phases I, II, and III were comparable between successful and unsuccessful subjects. In our protocol, GnRH antagonist was administered 1–2 days prior to blood collection of Phase II to prevent premature LH surge. However, serum LH levels were dramatically reduced in Phase II compared with Phase I only in the unsuccessful group not in the successful group. Furthermore, serum LH in Phase II had positive correlations with kisspeptin, E2 levels in Phase II and Phase III, and the number of RO, MII, and 2PN in the successful group. Serum kisspeptin levels in Phase II were positively correlated with the number of RO, MII, and 2PN only in the successful group but were positively correlated with serum P4 in Phase II in the unsuccessful group. These results indicate that the maintenance of serum LH levels in Phase II might have a positive effect on kisspeptin, E2, and outcomes of IVF/ICSI treatment. LH is a necessary hormone for synthesis of androgen, which then is aromatized to E2.[23] As a result, we postulated that higher LH levels in Phase II of successful subjects might increase androgen production leading to further synthesis of E2 and increased IVF/ICSI treatment outcomes. However, in unsuccessful subjects, the positive correlation between kisspeptin and P4 levels in Phase II suggests that there might be a shift of steroidogenesis into P4 in these patients resulting in poor outcomes. As a previous study showed that P4 levels measured on the day of hCG trigger (around 2–3 days after Phase II) had a negative association with the live birth rate.[45]

The present study showed that AMH levels at baseline (Phase I) were significantly higher in the successful group compared with the unsuccessful group even after adjustment for serum FSH at baseline (Phase I), which was in accordance with previous studies.[46,47] Furthermore, we found that serum AMH levels in Phase I were highly correlated with the numbers of RO, MII, and 2PN in the successful and unsuccessful groups implying that high AMH at baseline was positively associated with the outcomes of IVF/ICSI treatment. Our results were consistent with a previous study showing that AMH levels had a positive correlation with the number of RO.[48] Remarkably, although AMH in Phase I as well as kisspeptin in Phase II and Phase III had positive correlations with the outcomes of IVF/ICSI treatment, the relationships were different in some aspects. First, AMH showed positive correlations with the number of RO, MII, and 2PN in both successful and unsuccessful subjects with higher correlation coefficient values ($R = 0.627 - 0.890$) while kisspeptin levels in Phase II/III showed positive correlations with the number of RO, MII, and 2PN only in the successful group with lower correlation coefficient values ($R = 0.445 - 0.728$). Second, AMH did not have a significant correlation with hCG while kisspeptin in Phase II ($R = 0.636$, $P = 0.048$) and Phase III ($R = 0.717$, $P = 0.030$) showed significant positive correlations with hCG only in the successful group. Thus, our study supports that AMH is still the best predictor of the outcomes of IVF treatment because it had high correlations with the outcomes of IVF/ICSI treatment in both groups. However, as AMH is the indicator of diminished ovarian reserve (DOR)[49] which could not reverse in aging females, it might not be applicable in IVF/ICSI treatment. On the other hand, although kisspeptin might not be a good predictor of the IVF treatment because the levels were not different between successful and unsuccessful groups at the baseline (Phase I) timepoint, it could probably be applied in clinical practice to improve outcomes of IVF treatment including hCG levels.

Basal FSH concentrations have been screened routinely prior to the initiation of IVF treatment.[50] The current study showed that FSH levels in Phase I (basal FSH) were statistically lower in the successful group compared with the unsuccessful group even after adjusting for AMH at baseline (Phase I). In addition, FSH levels in Phase I had negative correlations with the number of RO, MII, and 2PN in total and unsuccessful subjects as well as the number of MII and 2PN in successful subjects. These results were consistent with previous studies reporting that elevated basal FSH levels were associated with poor outcome of IVF treatment[51–54] and have also been clinically used as a marker for DOR.[55] Furthermore, our results showed that basal FSH (Phase I) levels had a trend to be negatively correlated with E2 in Phase II in the successful group suggesting that high basal FSH was related to a reduction in E2 in Phase II. It is known that FSH is a necessary hormone to recruit and stimulate the growth of follicles.[56] However, high basal FSH levels might be caused by a reduction of inhibin B[57] and poor responsiveness of ovarian cells, for example from the aging process.[58] Inhibin B is synthesized by healthy granulosa cells of follicles[56,59] and exerts an

inhibitory effect on FSH secretion at the early stage of follicular development.[56] In DOR and the aging process, inhibin B levels were reduced resulting in decreased the inhibitory effect on FSH and thus increasing FSH levels.[59,60] Furthermore, in the aging process, granulosa cells have poor response to FSH, which is associated with lower *FSHR* expression in granulosa cells,[61] causing a reduction of E2 synthesis.[62] Subsequently, a reduction of a negative feedback of E2 results in an elevation of FSH levels.[62]

Conclusions

Kisspeptin levels in FF in Phase III, serum AMH levels in Phase I, and the number of RO, MII, 2PN, and transferred embryos as well as serum hCG levels were significantly higher but serum FSH levels in Phase I were significantly lower in the successful group compared with the unsuccessful group. The levels of kisspeptin and E2 in Phase III were maintained in the successful group but were significantly lower compared with Phase I in the unsuccessful group. The levels of kisspeptin and E2 in Phases II and III as well as LH in Phase II had positive correlations with outcomes of IVF/ICSI treatment in the successful group but not in the unsuccessful group. Furthermore, kisspeptin levels in FF were significantly higher than its levels in serum in the successful group. FF kisspeptin had a positive correlation with E2 in Phase II. Kisspeptin enhanced *CYP19A1* (aromatase) mRNA expression and aromatase protein concentrations in supernatant. We postulated that granulosa cells of successful subjects might be able to secrete higher levels of kisspeptin than unsuccessful subjects, which might be related to increased *CYP19A1* expression and secretion probably leading to maintenance of levels of serum E2 in Phase III. As a result, kisspeptin and E2 in Phase II and Phase III were positively associated with the outcomes of IVF/ICSI treatment. Further studies regarding supplementation of kisspeptin in Phase II/III could reveal the beneficial effects of kisspeptin on IVF/ICSI treatment.

Limitations

First, the sample size of the human study, especially in the successful group, is small. This could limit the significance of results in comparisons between successful and unsuccessful groups and in correlation analyses in the successful group even high correlation coefficient values were observed. Second, E2 levels in FF were not measured. As a result, the direct association between FF levels of kisspeptin and E2 could not be determined. Third, in the KGN cell study, E2 concentrations in supernatant were not measured. So, we could not confirm whether kisspeptin treatment increases estrogen synthesis/secretion.

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AUTHORS' CONTRIBUTIONS

LQ was engaged in conception, study design, sample collection, data acquisition, lab experiments, data analysis and interpretation, article writing, and final approval of manuscript. CSitticharoon was engaged in conception, study design, lab experiments, data acquisition, data analysis and interpretation, article writing, and final approval of manuscript. SP was engaged in conception, study design, patients' recruitment and consent, clinical examination, sample collection, data interpretation, and final approval of manuscript. IK was engaged in patient consent, sample collection, data acquisition, lab experiments, and final approval of manuscript. RS was engaged in patient consent, sample collection, data acquisition, lab experiments, and final approval of manuscript. PM was engaged in patient consent, sample collection, data acquisition, lab experiments, and final approval of manuscript. MC was engaged in lab experiments and final approval of manuscript. CSripong was engaged in conception, lab experiments, and final approval of manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The study protocol of IVF/ICSI treatment was approved by the Siriraj Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (Si 241/2017) in full compliance with international guidelines for human research protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines, and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP). All subjects signed the informed consents before the study. The protocols for the KGN cell study (116/2560 (Exempt)) were exempted from the Siriraj Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

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