Original Article

Positive Correlation of Oestradiol Level on Trigger Day with the Secretion Level of Endometrial Kisspeptin and Leukaemia Inhibitory Factor in the Mid-Luteal Stimulated Cycle

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Abstract

Background: Kisspeptin plays a role in the oestradiol negative-feedback regulation of GnRH as well as gonadotropin. In addition, kisspeptin has been postulated to induce the production of an important cytokine called leukaemia inhibitory factor (LIF). **Aims:** This study aims to investigate the correlation between varying oestradiol levels measured on trigger day of the ovarian stimulation and the mRNA expression level of endometrial kisspeptin and LIF. **Study Setting and Design:** Prospective cross-sectional study took place in Morula IVF Jakarta clinic. **Materials and Methods:** A total of 43 infertile couples underwent an in-vitro fertilization (IVF) program. Subjects were grouped based on oestradiol levels as follows: group A ([≧ 3000 pg/mL, n = 15], group B [2000–2999 pg/mL, n = 14], group C [<2000 pg/mL, n = 14]). **Statistical Analysis Used:** ANOVA test was utilised to compare the expression of kisspeptin and LIF among study groups while Pearson correlation was used to identify the correlation between variables. **Results:** A significantly higher mRNA expression of both Kisspeptin and LIF was found in group A than in groups B and C ($P < 0.001$). The mRNA expression of kisspeptin and LIF correlated positively with the oestradiol level ($r = 0.638$, $P < 0.001$ and $r = 0.634$, $P < 0.001$, respectively). Moreover, a strong association between Kisspeptin and LIF expression was also detected ($r = 0.700$, $P < 0.001$). **Conclusions:** mRNA expression of kisspeptin and LIF was significantly different according to the oestradiol levels in the study groups. Increased oestradiol level was shown to elevate the expression of endometrial kisspeptin and LIF in women undergoing the IVF programme.

Keywords: *IVF, kisspeptin, leukaemia inhibitory factor, oestradiol*

Introduction

Oestradiol has been shown to affect the expression of kisspeptin and its signalling system. In addition, a complex interplay of oestradiol and leukaemia inhibitory factor (LIF) is observed in which an increased oestradiol level results in an up-regulation of LIF expression in the endometrium at the implantation window.[1] LIF is a cytokine that has a potentially important role in regulating endometrial receptivity by promoting the anchoring trophoblasts, as indicated in several human and animal studies.[1-4]

A profound correlation between kisspeptin signalling and the expression of LIF has been demonstrated.

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The correlation was first discovered in a mouse model study.[5] Calder and Colleagues demonstrated that the embryos transferred into Kiss1$^{-/-}$ female mice failed to achieve implantation. While the administration of exogenous supplementation of gonadotropin and oestradiol attained successful ovulation, fertilisation and supported the pre-implantation embryo development, the mice failed to achieve pregnancy despite receiving progesterone supplementation. Ultimately, the study suggested that the perturbation of maternal implantation site could be caused by the lack of kisspeptin signalling. Furthermore, administering exogenous LIF in a uterus with low LIF expression was demonstrated to partially rescue the implantation process, suggesting the direct effect of kisspeptin on the expression of LIF in uterine glands. This study aimed to become the first to evaluate the relationship of varying oestradiol levels on trigger day of a controlled ovarian stimulation with the level of endometrial kisspeptin and LIF expression in infertile patients undergoing a frozen IVF cycle.

Subjects and Methods

Patient selection

This observational cross-sectional study was performed in Morula IVF Jakarta Clinic. Signed informed consent was received from all studied subjects. This study has adhered to Helsinki Declaration (2013). The inclusion criteria for subject selection included women who underwent an IVF programme with an antagonist stimulation protocol and a freeze-all cycle. The exclusion criteria were as follows: Women with endometriosis or adenomyosis, endometrial polyp, uterine myoma, hydrosalpinx, a history of myomectomy, adenomyosis resection and recurrent curettage for at least three times. A total of 43 patients that met the eligibility criteria were enrolled from September 1, 2020 to December 30, 2020. To investigate the correlation between the varying oestradiol level and the mRNA expression of Kisspeptin and LIF, the subjects were grouped based on the oestradiol levels on trigger day as follows: Group A ([≧ 3000 pg/mL, n = 15], group B [2000-2999 pg/mL, n = 14], group C [<2000 pg/mL, n = 14]). The cut-off used to create the groups was based on our previous study.[6] This research has received ethical approval from the Medical Research Ethics Committee of the Faculty of Medicine, UNHAS on September 4, 2020, No:519/UN4.6.4.5.31/PP3/2020.

Treatment protocol

Subcutaneous injection of gonadotropin (Gonal F (Merck, Serono) or Menophur (Ferring, Switzerland) was initiated at day 2 or 3 of the menstrual cycle. The starting dose varied between 150 and 375 IU according to the patient's clinical characteristics such as hormonal basal profile, anti-Mullerian hormone (AMH), and antral follicle count (AFC). After four daily primings, the follicular growth was analysed through ultrasonography and the oestradiol level was measured to consider if dosing adjustment was necessary. 0.25 μg of GnRH antagonist cetrorelix (Cetrotide (Merck, Serono) was administered when the follicle had reached 11–12 mm. Maturation trigger using 250 μg/6500 IU of Ovidrel (Merck, Serono) was given when at least 3 follicles had reached 18 mm. Ovum pick-up (OPU) procedure was conducted 36 h following Ovidrel injection. All mature oocytes were inseminated through intra-cytoplasmic sperm injection (ICSI) or intra-morphologically selected sperm injection (IMSI) procedure. Fertilised oocytes were then cultured up to day 5 and top-quality blastocysts were frozen for the frozen embryo transfer cycle.

Endometrial sample collection

Endometrial tissue sample biopsy was collected on day 5 following the OPU procedure (LH + 7) using Pipelle endometrial suction catheter where the patient was awake with no sedation. After cleansing the vaginal area, patients were positioned in a lithotomy position. A speculum was inserted to make way into the uterus-cervix area. The anterior cervical lip was grasped and the Pipelle catheter was gently inserted into the uterus cavity through the internal cervical ostium until it reached the uterine fundus. Negative pressure or suction was obtained by pulling the internal piston out of the catheter tube in a continuous motion. The catheter was then slowly drawn back and forth within the uterine cavity in a spiral motion to aspirate the endometrial tissue. Collected samples in the proximal Pipelle catheter were then immediately flushed with L6 buffer (Fluka Chemie AG, Buchs, Switzerland) as the transport medium using a syringe. The tissue samples were then processed for RNA extraction.

RNA extraction and quantitative reverse transcription-polymerase chain reaction

RNA extraction from the endometrial samples was performed according to Boom method.[7] Reverse transcription of the extracted RNA was conducted using a commercial kit (Promega, USA). Quantitative PCR of the resultant cDNA, for quantifying kisspeptin, LIF, and GDPH expression, was executed according to the one-step Tomomi Yajima protocol utilizing SYBR green PCR master mix. This protocol was optimised using a real-time PCR machine CFX Connect System (USA). Specific primers used in the study are summarized in Table 1. Human glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal control for the quantitative reverse transcription-polymerase chain

Table 1: Primer sequences utilised in the study

Primer	Sequences (5' to 3')
Kisspeptin sense (forward)	CCATTAGAAAAGGTGGCCTCTGT
Kisspeptin antisense (reverse)	ACGGCTCAGCCTGGCAGTAG
LIF sense (forward)	ACAGAGCCTTTGCGTGAAAC
LIF sense antisense (reverse)	TGGTCCACACCAGCAGATAA
Human GAPDH for kisspeptin (forward)	GTCTCCTCTGACTTCAACAGCG
Human GAPDH for kisspeptin (reverse)	ACCACCCTGTTGCTGTAGCCAA
Human GAPDH for LIF (forward)	GGGAGCCAAAAGGGTCATCATCTC
Human GAPDH for LIF (reverse)	CCATGCCAGTGAGCTTCCCGTTC

LIF=Leukemia inhibitory factor, GAPDH=Glyceraldehyde-3-phosphate dehydrogenase

reaction (PCR). Overall, the PCR comprised 40 cycles of denaturation at 95°C for 1 min followed by annealing for 20 s at 54°C.

Statistical analysis

The measurement of data distribution normality was performed using Kolmogorov–Smirnov. SQRT method was used to normalize the data distribution. Baseline and clinical characteristics of the subject were presented as mean ± standard deviation or median (min-max) according to the data distribution. Categorical data were presented as percentages (n (%)). A minimum of 14 subjects was required in each oestradiol group to have a statistical power of 80%. Bivariate analysis between study groups was executed using the ANOVA test. Calculating the relative expression level of each target gene was accomplished using the Delta-Delta Ct equation ($2^{-\Delta\Delta Ct}$). $P < 0.05$ was considered statistically significant.

Results

Baseline and clinical characteristics of the study subjects

The subjects were equally distributed among the three oestradiol groups. The baseline and clinical characteristics of patients are summarised in Table 2. Statistically significant differences in female age and unexplained infertility factors were observed among the groups. The mean of female age was highest in group B followed by group C and A. Unexplained infertility proportion was also higher in group B than that of group C and A. Other parameters of the baseline characteristics were comparable among the study groups. Several clinical characteristics such as AMH, AFC, basal LH, progesterone level on the trigger day, number of follicles, and number of retrieved oocytes were statistically different among the groups. Elevated oestradiol level was positively correlated with a higher median value of those variables as observed in group A compared to groups B and C.

Correlation of oestradiol level with the expression of Kisspeptin and leukemia inhibitory factor mRNA

Increased mRNA expression of kisspeptin and LIF were shown to correlate with higher oestradiol levels on the trigger day [Table 3]. A significantly higher mRNA expression of both kisspeptin and LIF was found in group A than those of group B and C. Pearson correlation measurement indicated the positive correlation between the oestradiol level and the expression level of kisspeptin and LIF ($r = 0.638$, $P < 0.001$ and $r = 0.634$, $P < 0.001$, respectively) [Figure 1]. Furthermore, a strong association between the expression of Kisspeptin and LIF was also detected ($r = 0.700$, $P < 0.001$) which would imply the direct impact of an elevated Kisspeptin on the enhanced expression of LIF [Figure 2].

Identifying potential confounders was initiated to further establish the relationship between oestradiol level and mRNA expression of kisspeptin and LIF. After adjusting for AMH, basal FSH, and infertility duration through multivariable linear regression analysis, a significant correlation between oestradiol level and kisspeptin expression persisted, indicating the true relationship between these two variables (coefficient correlation 0.788, $P < 0.001$). A similar trend was also observed between the oestradiol level and LIF expression after adjusting for potential confounders (coefficient correlation 0.763, $P < 0.001$).

Discussion

This study managed to demonstrate the positive correlation between oestradiol level and the expression of kisspeptin and LIF mRNA. This study also suggested a complex interaction between oestradiol, kisspeptin, and LIF in supporting the implantation process. Oestradiol has been shown to control the kisspeptin system which primarily functions in the hypothalamus.[8,9] The positive correlation found in this study corroborates the essential role of kisspeptin during implantation as demonstrated previously by Jamil *et al*.[10] In their study, the serum kisspeptin expression level on human chorionic gonadotropin (HCG)-day was higher in the pregnant group than in the non-pregnant group ($P < 0.001$). The elevated serum oestradiol level in women undergoing IVF programme has also been proposed to influence the expression of kisspeptin mRNA in the endometrial stroma at the late secretion phase (5 days after ovum pick up procedure).[11]

Table 2: Baseline and clinical characteristics of subjects in the study

Parameters	Group A (*n*=15)	Group B (*n*=14)	Group C (*n*=14)	*P*
Baseline characteristics				
Female age (year) [a]	31.67±5.09	36.57±4.59[d]	34.93±4.89	0.030
Type of infertility[c], *n* (%)				
Primary	13 (86.7)	11 (78.6)	10 (71.4)	0.601
Secondary	2 (13.3)	3 (21.4)	4 (28.6)	
Infertility duration (year) [b]	7 (2-18)	5.5 (3–12)	5 (2-13)	0.829
Body mass index (kg/m2) [a]	24.13±4.17	23.36±3.79	23.57±5.17	0.890
Infertility cause[c], *n* (%)				
Sperm factor	11 (73.3)	4 (28.6)	8 (57.1)	0.051
Female factor	6 (40)	2 (14.3)	6 (42.9)	0.204
Combination	1 (6.7)	3 (21.4)	1 (7.1)	0.379
Unexplained	3 (20)	10 (71.4)	4 (28.6) [e]	0.011
Clinical characteristics				
AMH (ng/mL) [b]	4.7 (1.96-40)	1.9 (0.70-3.92) [d]	1.15 (0.15-5) [d]	<0.001
AFC[b]	15 (9-20)	7.5 (4-16) [d]	6.5 (1-10) [d]	<0.001
Basal hormones				
Basal FSH (mIU/mL) [b]	6.5 (3.30-9.90)	7.15 (4.60-19)	7.85 (6.20-11.91)	0.083
Basal LH (mIU/mL) [b]	6 (3.90-12.60)	4.3 (2.80-9.80)	4 (2.30-7.20) [d]	0.011
Basal oestradiol (pg/mL) [a]	35.81±11	41.07±10.80	41.32±17	0.451
Basal progesterone (ng/mL) [b]	0.14 (0.05-0.35)	0.17 (0.05-1.08)	0.18 (0.05-0.50)	0.366
Total dose of FSH (IU) [b]	1800 (1200-3225)	2400 (1800-3525)	2700 (1350-4125)	0.053
Stimulation duration (day) [b]	9 (8–10)	8 (8-11)	9 (8-11)	0.411
Progesterone level on the trigger day (ng/ml) [a]	0.74±0.30	0.54±0.30	0.46±0.26[d]	0.031
Endometrial thickness (mm) [b]	12 (8–14)	11 (9-15)	10 (7.5-13)	0.295
Number of follicles[b]	14 (8–51)	8.50 (5-16) [d]	6.5 (1-16) [d]	<0.001
Number of retrieved oocytes[b]	13 (8–48)	7 (5-11) [d]	4.5 (1-16) [d]	<0.001

[a]Data are presented as mean±SD, [b]Data are presented as median (minimum-maximum), [c]Data are presented as number of subjects and percentage *n* (%), [d]Compared with Group A, *P*<0.05; [e]Compared with Group B, *P*<0.05. Kruskal-Wallis test or ANOVA test was used depending on the normality of the data. The Chi-square test was used for categorical variables. AMH=Anti müllerian hormone, AFC=Antral follicle count, FSH=Follicle-stimulating hormone, LH=Luteinizing hormone

Table 3: Relative expression of kisspeptin and leukemia inhibitory factor mRNA according to the oestradiol level on the trigger day

Gene target	Group A (*n*=15)	Group B (*n*=14)	Group C (*n*=14)	*P*
Kispeptin	10.28±0.83[bc]	8.27±0.98[ac]	7.13±0.68	<0.001
LIF	12.44±0.81[bc]	11.47±0.77[ac]	9.50±0.79	<0.001

[a]Compared with Group A, *P*<0.05; [b]Compared with Group B, *P*<0.05;[c]Compared with Group C. LIF=Leukemia inhibitory factor

Correlation between the serum oestradiol levels and both the serum and follicular fluid kisspeptin was also investigated by Rehman and colleagues[12] at multiple stages of the IVF programme namely the follicular phase, maturation trigger, ovum-pick up, and embryo transfer day. The study revealed a gradual increase in oestradiol and kisspeptin level which began at the follicular phase up to the OPU day and that it might contribute to the optimization of endometrial thickness, oocyte fertilizability, and clinical pregnancy.

Oestradiol also affects the secretion of LIF.[1,4] Our study observed a positive correlation between these two variables in which a higher oestradiol level on HCG-day gave rise to the elevated endometrial LIF expression. A 2002 study conducted by Hewitt *et al.*[13] exhibited the rescue of decidualization and implantation by supplementing oestrogen receptor knockout mice with exogenous LIF implying the regulatory function of oestradiol on LIF expression. As LIF is a prominent cytokine that regulates trophoblast invasion and has been shown to correlate positively with elevated oestradiol, this finding supports previous studies that increased oestradiol level did not impair the clinical pregnancy rate.[14-17]

A strong association between kisspeptin and LIF expression was also noticed in our study, which supports the previous result.[5] Utilizing mice as a model, Calder and Co-workers showed that implantation failure was observed in *Kiss1*$^{-/-}$female mice. A weak expression of LIF in the uterine gland of the *Kiss1*-/-mice was a proposed mechanism for the implantation failure. Administering exogenous LIF was then proven to partially restore the

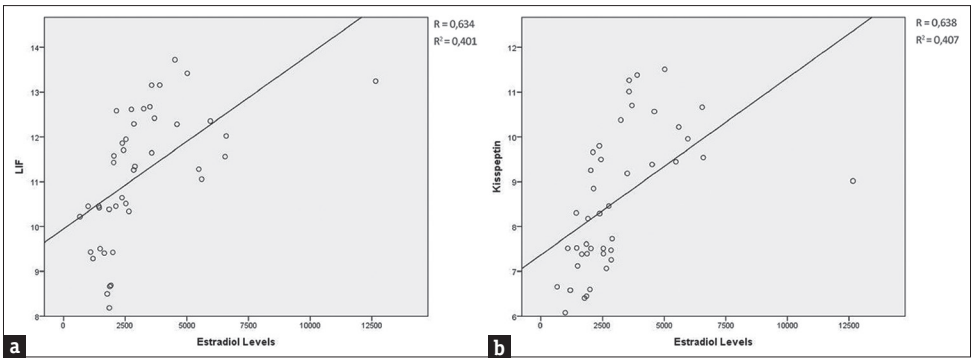

Figure 1: Correlation between oestradiol level and mRNA expression of (a) leukemia inhibitory factor and (b) kisspeptin

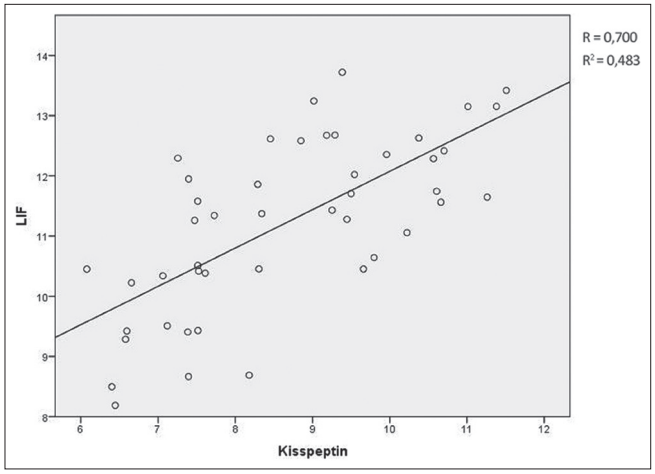

Figure 2: Correlation of mRNA expression between kisspeptin and leukemia inhibitory factor

implantation process which implied the prominent roles of kisspeptin signalling, as well as oestradiol, in regulating the expression of LIF in the uterine glands.

This study is limited in the presented results which only measured the interrelation between independent and dependent variables without assessing the important outcomes of the IVF programme such as implantation rate, clinical pregnancy rate, and live birth rate.

In conclusion, the oestradiol level in a controlled ovarian stimulation of IVF was shown to positively correlate with the expression of kisspeptin and LIF mRNA. Increased oestradiol levels on the trigger day appeared to enhance the expression of both kisspeptin and LIF. The strong positive correlation between kisspeptin and LIF also signifies the critical function of kisspeptin in regulating the expression of LIF in the uterus glands.

Data availability statement

Data are available upon reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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