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, endometriosis or adenomyosis, endometrial polyp, 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 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.[6] 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 reaction.
Table 1: Primer sequences utilised in the study

| Primer | Sequences (5’ to 3’) |
|--------|----------------------|
| Kisspeptin sense (forward) | CCATTGAAAAGGTGGCCTCTGT |
| Kisspeptin antisense (reverse) | ACGGCTCAGCCTGGCAGTAG |
| LIF sense (forward) | ACAGAGCCCTTGGCGTAAAC |
| LIF sense antisense (reverse) | TGGTCCACACCAGCAGATAA |
| Human GAPDH for kisspeptin (forward) | GTCTCTCTGACTTCACACAGC |
| Human GAPDH for kisspeptin (reverse) | ACCACCTGTGGCTGATGCC |
| Human GAPDH for LIF (forward) | GGGAGCAAAAGGGTCATC |
| Human GAPDH for LIF (reverse) | CCATTAGAAAAGGTGGCCTCT |
| LIF sense antisense (forward) | CCATTAGAAAAGGTGGCCTCT |
| LIF sense antisense (reverse) | CCATTAGAAAAGGTGGCCTCT |

**Table 2.**

| Primer | Sequences (5’ to 3’) |
|--------|----------------------|
| LIF sense (forward) | ACAGAGCCCTTGGCGTAAAC |
| LIF sense antisense (reverse) | TGGTCCACACCAGCAGATAA |
| Human GAPDH for kisspeptin (forward) | GTCTCTCTGACTTCACACAGC |
| Human GAPDH for kisspeptin (reverse) | ACCACCTGTGGCTGATGCC |
| Human GAPDH for LIF (forward) | GGGAGCAAAAGGGTCATC |
| Human GAPDH for LIF (reverse) | CCATTAGAAAAGGTGGCCTCT |

**Table 3.**

- **Correlation of oestradiol level with the expression of kisspeptin and LIF 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. The positive correlation found in this study corroborates the essential role of kisspeptin during implantation as demonstrated previously by Jamil et al. 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).
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)   | 31.67±5.09    | 36.57±4.59d   | 34.93±4.89    | 0.030 |
| Type of infertility, 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) |          |               |               |       |
|                     | 7.2 (2.18)    | 5.5 (3–12)    | 5 (2–13)      | 0.829 |
| Body mass index (kg/m²) |    |               |               |       |
|                     | 24.13±4.17    | 23.36±3.79    | 23.57±5.17    | 0.890 |
| Infertility cause, 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)      | 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)    | 6.5 (1-10)    | <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 | 41.52±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) |       |
| 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 a | 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 |

aData are presented as mean±SD, bData are presented as median (minimum-maximum), cData are presented as number of subjects and percentage n (%), dCompared with Group A, P<0.05; eCompared with Group B, P<0.05. Kruskal-Wallis test or ANOVA test was used depending on the normality of the data. 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     |
|-------------|---------------|---------------|---------------|-------|
| Kisspeptin  | 10.28±0.833ac | 8.27±0.988ac  | 7.13±0.68     | <0.001|
| LIF         | 12.44±0.811ac | 11.47±0.777ac | 9.50±0.79     | <0.001|

aCompared with Group A, P<0.05; bCompared with Group B, P<0.05; cCompared 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 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. 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. 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.
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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