

## LIF and LIF-R Protein Expression in *Macaca nemestrina* Midluteal Endometrial Tissues After Controlled Ovarium Stimulation

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### Abstract

Implantation rate in In Vitro Fertilization (IVF) as assisted reproductive technology has been remained low and suspected being caused by the negative effect of 7controlled ovarium stimulation (COS) procedure. Several studies reported that the negative effect made the maturity of endometrium patient, one of the factors was seen by biomolecular changes, with IVF occurs faster than normal.

In this study, leukemia inhibitory factor (LIF) and the receptor determined in tissue endometrial of *M. nemestrina* to analyze the expression of them after COS. *M. nemestrina* stimulated with Gonadotropin Releasing Hormone (GnRH) and recombinant follicle stimulating hormone (r-FSH) using agonist protocol. There were two groups of r-FSH (4 samples in 30IU dose group and 4 samples in 50IU dose group) that used and compared the effect with 3 samples as the control group. A concentration of estrogen (E2) and progesterone (P4) from the late-follicular phase also determined to see the correlation with them. LIF and LIF-R concentration determined by ELISA assay and localization of expression determined by IHC.

Statistic analyzed by One-way ANOVA test for normal distribution and Kruskal Wallis for not-normal distribution to compare LIF and LIF-R within dose group. Spearman test used to analyze the correlation of estrogen and progesterone with LIF and LIF-R. We found no statistically significant of LIF and LIF-R within group. Only correlation of progesterone and LIF-R that have statistically significant which the correlation was strong in a negative direction. Expression of LIF found in cytoplasmic and that was statistically significant within group. The expression in r-FSH groups were lower than a control group. Expression of dose LIF-R found in cytoplasmic and there was no statistically significant within dose group.

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### Introduction

Infertility in married couples is defined as no pregnancy after 1 year of regular sexual intercourse without using contraceptives and is one of the multiple reproductive problems encountered by modern couples.<sup>1</sup> Interventions for infertility have progressed with recent scientific and technological developments.

Assisted reproduction technology (ART) is a promising intervention for young infertile couples, and for those who are older than 38 years of age. With increases in the standard of living of human communities, ART is increasingly considered, despite its high cost.

Under the conditions of ART, implantation becomes possible when the embryo and the endometrium are synchronized, and normal blastocyst embryos and receptive endometrium are required.<sup>2-5</sup> Conversely, poor synchronization of the embryo and endometrium during the embryo transfer process is a suspected cause of implant failure in the *in vitro* fertilization (IVF) patients. To achieve IVF, acquired embryos develop according to developmental times, but not with the

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endometrium<sup>8</sup>. Several studies also indicate that changes in the maturation conditions of the endometrium in IVF patients lead to faster endometrial maturation, and observations of the developmental stages have been confirmed in both histological and molecular studies.<sup>6-10</sup>

Leukemia inhibitory factor (LIF) has been used as a biomarker to determine the optimal implantation times and is crucial in endometrial maturation. LIF is a pleiotropic cytokine of the interleukin-6 (IL-6) family and was the first cytokine to be associated with the implantation process. LIF regulates various implantation processes, including the development of receptive endometrium, interactions between the endometrium and blastocysts, decidualization of stromal cells, blastocyst invasion, blastocyst development, and uterine leukocyte infiltration. LIF also regulates the synthesis of prostaglandins (PGs), which contribute to the implantation and decidualization processes<sup>14-16</sup>.

LIF binds the LIF receptor (LIF-R), which is present on the cell membranes of the endometrium and the embryo. In the endometrium, LIF-R is a heterodimer of gp130 receptor and LIF-R alpha (LIF-R $\alpha$ ) subunits. Like LIF, appropriate LIF-R expression levels are required for implantation during the time of implantation. Specifically, LIF-R expression is increased after ovulation and is sustained until the end of the menstrual cycle. LIF-R expression is maximal between the 19th and 25th days of the menstrual cycle, equating with post-ovulation days 5–10<sup>17,18</sup>.

To the best of our knowledge, LIF and LIF-R expression levels have not been studied in relation to concentrations of the reproductive hormones estrogen (E2) and progesterone (P4) following the treatments with gonadotropin releasing hormone GnRH agonists. Therefore, we compared the concentrations of LIF and LIF-R in GnRH treatment animals with those under normal conditions and correlated these with the endogenous concentrations of E2 and P4.

## Materials and methods

Enzyme-linked immunosorbent assays (ELISA) and Immunohistochemistry (IHC) were performed at the Biochemistry Laboratory Faculty of Medicine and Wet Laboratory Universitas Indonesia between January and August 2018. All the experimental protocols were approved by the

Research Ethics Committee of Faculty of Medicine Universitas Indonesia; 262 / UN2.F1 / ETIK / III / 2018.

## Samples

Samples were a stored biological material, frozen endometrial tissue and paraffin-embedded sections of endometrial tissue, from the previous experimental originates from stimulated adult *Macaca nemestrina* primates of reproductive age (8–10 years of age; weight, 5–8 kg) with a history of having an offspring. *Macaca* were grouped in two, *Macaca* with stimulation and without stimulation as a control. In previous experimental, a stimulation group were stimulated with GnRH agonist for 14 days (during the mid-luteal phase) and recombinant follicle stimulating hormone (r-FSH) for 10 and/or 12 days (during the final follicular phase). GnRH agonist treatments were applied at 30IU and 50IU of doses. There were 11 number of samples in each samples form, frozen endometrial tissue and paraffin-embedded section, that contain of three groups; 3 samples in control group, 4 samples in 30IU of dose group, and 4 samples in 50IU of dose group. The data of E2 and P4 concentration used from the previous experimental with the same sample and protocol with this research.

## Assay

### Enzyme-linked immunosorbent assays (ELISA)

Frozen tissue was homogenized in 500 $\mu$ l phosphate buffered saline 0,01M (PBS; pH 7,4) using *micrometer* and *tissue homogenizer*. Tissue homogenate that had been formed then added with 500  $\mu$ l PBS 0,01 M and centrifuged in 5000g for 10 minutes to get the supernatant.

Using the tissue homogenate, a concentration of LIF was carried out with Monkey LIF ELISA Kit (Elabscience; E-EL-MK0181) according to manufacturer's protocol. The sensitivity of the assay was 18,75 pg/ml. A concentration of LIF-R was determined using Monkey LIF-R ELISA Kit (MyBioSource; MBS735084) according to manufacturer's protocol with sensitivity 0,1 ng/ml.

### Immunohistochemistry (IHC)

The expression levels of LIF and LIF-R in endometrium were analyzed by immunohistochemistry on a paraffin-embedded section. Antigen retrieval was performed using

One step Neopoly Polymer Detection kit protocol. The first treatment was with Tris EDTA pH 9 for 15 min in Retrieval Generation One BioGear technology. Section than cooled down at room temperature for 45 minutes with still in Tris EDTA then washed with PBS. Monoclonal human anti-LIF antibody (AF-250-NA; R&D System R&D) in 1:30 dilution was added for 24 h at 4°C in first fifteen sections, and the second fifteen sections were added with Polyclonal monkey anti-LIF-R (ab101228; Abcam) in 1:250 dilution for 1 h at room temperature. Section was then washed with PBS and incubated with HRP for 30 min. The antigen-antibody complexes were visualized using DAB and counterstained with hematoxylin.

Five images each sample in 40x magnitude from each group were taken with the microscope setting unaltered. The images than analyzed using imageJ software to get optical density score. The score than calculate in average of each groups.

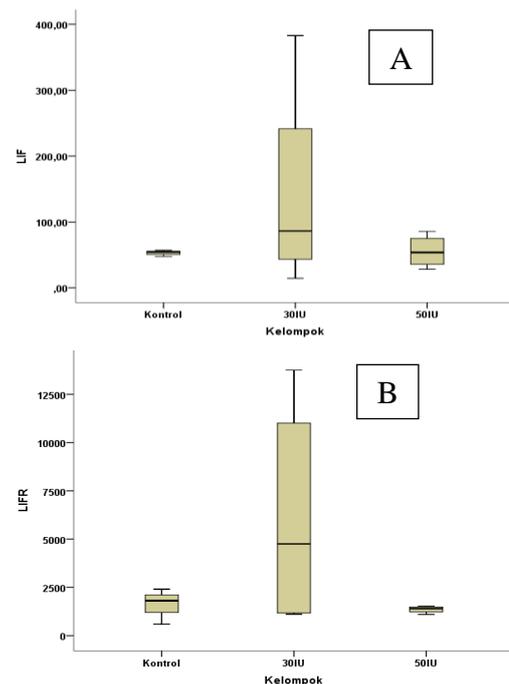
### Statistical analysis

All quantitative data were analyzed using SPSS 23. The concentration of LIF and LIF-R determined using one tail ANOVA to analyze the association within dose group in normal data and Kruskal-Wallis in not-normal data. Post-hoc used if signification found in the result. Correlation of E2 and P4 to LIF and LIF-R was analyzed using Spearman.  $P \leq 0,05$  was considered significant.

### Results

#### A Concentration of LIF and LIF-R and the correlation with steroid

The concentration of LIF and LIF-R, resulted from ELISA test, analyzed to compare them within group. In this data, median used because the data was not normally distributed. The concentration of LIF in control group was 54,21 pg/ml. In stimulation groups, concentration was 86,32 pg/ml in 30IU dose group and 53,75 pg/ml in 50IU dose group. The concentration of LIF-R in control group was 1811 pg/ml where in 30IU dose group was 4753 pg/ml and 1404 pg/ml in 50IU dose group. Figure 1 shows that no statistically different found in concentration of LIF and LIF-R within groups (p value LIF 0,523; p value LIF-R 0,695).



**Figure 1. LIF and LIF-R concentration based on group dose.** Concentration LIF within group was not statistically different (p value 0,523) (A), concentration LIF-R within group also not statistically different (p value 0,695). Statistic analytical used *Kruskal-Wallis*.

E2 had positive correlation with LIF but not significantly difference (r: 0,127; p value: 0,355). P4 also had not significantly difference but with negative direction (r: -0,382; p value: 0,123). E2 had moderate correlation with LIF-R but not significantly difference (r: -0,382; p value: 0,123). Correlation with significant difference found in correlation of P4 and LIF-R. The correlation was strong in negative direction (r: -0,597; p value: 0,026) (Table 1).

Variable	N	R	p value
E2			
LIF	11	0,127	0,355
LIF-R	11	-0,382	0,123
P4			
LIF	11	-0,164	0,315
LIF-R	11	-0,597	0,026

**Table 1.** Correlation of Estrogen with LIF and LIF-R.

Correlation of P4 and LIF-R than analyzed by separating the groups. The correlation with significantly difference found only

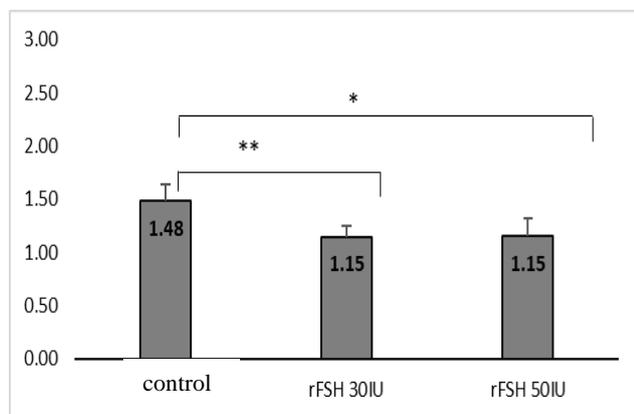
in rFSH groups. Table 2 shows that P4 had a significantly strong correlation with LIF-R in rFSH group where the direction was negative.

Variable	N	R	P value
Control	3	-0,866	0,333
rFSH group	8	-0,714	0,047

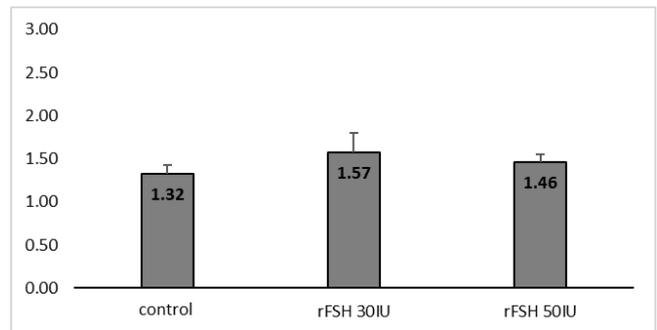
**Table 2.** Correlation of Progesterone with LIF-R by groups.

### Expression of LIF and LIF-R in tissue endometrial

LIF and LIF-R were expressed in the cytoplasm of glands, epithelial, and stroma. In this study, only LIF and LIF-R in glands and epithelia will be explained because some experiment shows that expression in stroma more relevant in decidualization event compared in implantation event. Expression of LIF found significant difference within group (average density score in control group  $1,48 \pm 0,164$ ; 30IU dose group  $1,15 \pm 0,104$ ; and 50IU dose group  $1,15 \pm 0,165$ ; p value p value 0,016) (Figure 2). Expression of LIF-R found no significantly difference within group (average density score in control group  $1,32 \pm 0,107$ ; 30IU dose group  $1,57 \pm 0,221$ ; and 50IU dose group  $1,46 \pm 0,088$ ; p value p value 0,114) (Figure 3).



**Figure 2.** The average of optical density LIF. There were significantly differences of LIF expression within group. The trend was decreased from control group to rFSH groups. Statistical analysis used ANOVA test with LSD to compare one group to another.



**Figure 3.** The average of optical density LIF-R. There were no significantly differences of LIF-R expression within group. Statistical analysis used ANOVA test.

### Discussion

In this study, tissue endometrial of *Macaca nemestrina* in mid-luteal phase was used. LIF and LIF-R concentration determined and found not significantly different within dose group. It concluded that dose of rFSH, not the variable that directly caused changes in this molecular concentration. A response the stimulation suspected as the main caused.<sup>11</sup>

rFSH was given to increase the number of mature follicles, more than the normal count, and known to cause an increase of E2 and P4 level, also more than the normal level. FSH is known to initiated secretion of E2 and P4 which the endometrium as the target organ from both of them.<sup>12</sup> E2 found had a high concentration in rFSH stimulation group but not significantly caused a different concentration of LIF and LIF-R. It was suggested that E2 was not the main regulator of LIF and LIF-R in *Macaca nemestrina*. E2 found significantly correlated with the concentration of LIF in mouse but not in a primate. P Paiva found that regulation of LIF expression was different in mouse and primate. P4 was the main regulator for LIF expression in a primate with direct and indirect regulation.<sup>13</sup>

Indirect regulation of P4 to activate LIF was in line with our research. Hong-Der Tsai et al also found that expression of LIF was not significantly different both in a woman with low and high P4 level.<sup>14</sup> Downregulation of progesterone receptor (PR) was suggested as confounding variable in this mechanism. Reduction of LIF expression in epithelial luminal endometrium after injection with antagonist PR (Mifepristone, RU486) strengthened this finding.<sup>13</sup> Hence synchronization of P4 and its receptor was needed to stimulate optimal expression of LIF.

Fertile woman with a normal mechanism, P4 found to stimulate expression of galectin 15, cathepsin L1, cystatin C, endothelial PAS domain protein 1, gastrin-releasing peptide, and insulin-like binding protein 1 and 3 where those genes needed to induce downregulation of PR. But, expression of LIF-R found an increase in this mechanism.<sup>15,16</sup> In this study, a P4 level in rFSH groups found significantly correlated with LIF-R, but the correlation was strong in a negative direction. This was suspected cause of the time limited of P4 and LIF expression. High level of P4 after stimulation (in proliferation phase) found to accelerate the timing of maturation, cause LIF expression occurred 10 days faster than its normal. Expression both would be decreased after reaching certain concentration caused by antagonist mechanism from both receptors. Furthermore, LIF and LIF-R suggested having paracrine and autocrine to stimulate each other.<sup>6-8</sup> By combined this mechanism, suggested that tissue endometrial sample already not in mid-luteal phase by day of the collection caused by acceleration on a timing of maturation. Therefore, LIF-R found in downregulation mechanism.

Expression of LIF in cytoplasm strengthened that suggestion. LIF found significantly low in rFSH groups than the control group and suggested as an antagonist mechanism of soluble LIF-R that increased after stimulated by a high level of P4. LIF-R found positive in the cytoplasm where the expression was higher in rFSH group than the control group, although not significantly different and in low positive staining. LIF - soluble LIF-R binding activate JAK-STAT3 but in different pathway compared LIF-membrane LIF-R. LIF-soluble LIF-R induces growth arrest and differentiation cells. MAPK signaling pathway also found activate and inhibit proliferation mechanism. Therefore, the condition of tissue endometrial in this study might not in a mature stage.<sup>17-18</sup>

### Conclusions

In summary, doses of rFSH were not the cause of molecular changes in endometrium. Direct effect of stimulation, in this study was P4 levels, caused it by controlling soluble LIF-R expression.

### Declaration of Interest

The authors report no conflict of interest.

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