Original Article

# Increased Estradiol Levels Effect on Leptin Expression in Midluteal Endometrial Tissue of Macaca Nemestrina (Southern Pig-Tailed Macaques)

**Nurhuda Sahar; Ph.D.[1], Adriana Viola Miranda; B.Med.[2], Afif Rasyad; B.Med.[2], Karina Rahmaningrum; B.Med.[2], Kusmardi Kusmardi; Ph.D.[3], Diyah Kristanty; M.Biomed.[4]**

1 Department of Biology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
2 Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
3 Department of Anatomical Pathology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
4 Department of Clinical Pathology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

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## Abstract

**Objective:** To measure the effect of serum estradiol (E2) levels on leptin expression in Macaca nemestrina (southern pig-tailed macaque) endometrial tissue.

**Materials and methods:** This study used paraffin-embedded midluteal phase endometrial tissue blocks of Macaca nemestrina from previous study. Included subjects were 15 female macaques of reproductive age (8-10 years) with a previous history of producing offspring, which were divided into four groups: groups administered with 30 IU, 50 IU, and 70 IU r-FSH (intervention group), and no r-FSH (control group). The stimulation was done following GnRH agonist long protocol. Staining was done using immunohistochemistry. Leptin expression was measured using immunohistochemistry (IHC) Profiler plugin of ImageJ software and counted semi-quantitatively as Histological Score (Hscore).

**Results:** Correlation between E2 concentration to stromal leptin expression was observed (p=0.043).

**Conclusion:** Serum estradiol concentration is found to be correlated with leptin expression in Macaca nemestrina, suggesting a mechanism of decreasing endometrial receptivity among women undergoing controlled ovarian hyperstimulation.

**Keywords:** In Vitro Fertilization; Controlled Ovarian Hyperstimulation; Leptin; Endometrial Receptivity

## Introduction

Controlled ovarian hyperstimulation (COH) is known to play a crucial role in In Vitro Fertilization and Embryo Transfer (IVF-ET) due to its ability to provide a high number of viable oocytes, thus compensating biological limitations and laboratory errors during further IVF-ET procedure (1). However, its effect on the hypothalamus-pituitary-ovarian (HPO) axis is adversely associated with endometrial receptivity (2). Several clinical studies suggest that supraphysiological levels of estradiol (E2) during midluteal phase are detrimental to embryonic implantation (3). Despite this knowledge, the causation of this effect remains controversial.

**Correspondence:**
*Adriana Viola Miranda*
*Email: adriana.viola@ui.ac.id*

One of the suggested mechanisms is the role of E2 in increasing leptin expression. Leptin is an adipokine hormone initially considered to regulate food intake and energy balance (4), but experimental evidence has shown that it is also crucial in various metabolic and endocrine processes, including reproductive function. Leptin negatively affects endometrial receptivity through its inhibitory role during the thickening of the endometrial lining, uterine fluid removal, and decidualization and maternal-fetal communication (5-7). Despite this knowledge, studies on estradiol regulation of leptin expression in endometrial tissue remain limited. This study aimed to observe the correlation between leptin expression levels in the midluteal phase endometrial tissue of Macaca nemestrina (southern pig-tailed macaques) and estradiol concentration.

## Materials and methods

***Subject characteristics:*** This is an experimental study. We used paraffin-embedded tissue blocks of a previous study involving the uterus of Macaca nemestrina (southern pig-tailed macaques). The species has a similar cycle and hormonal levels with humans: their cycle length and luteal phase length are approximately one-month and 15 days, respectively (8). Subjects included in this study are 16 female macaques of reproductive age (8-10 years, 10-15 kg) with a history of producing offspring. They were tattooed with an identification number in the groin area, housed in individual cages of stainless material in a dedicated room. All animals were quarantined and adapted to new individual cages for two to three menstrual periods. During this time, animal health was maintained and any treatment was administered as needed. In the final evaluation, one subject was excluded since its menstrual cycle failed to stabilize after 3 stimulation cycles.

The animals were obtained from the Primates Animal Study Center of the Bogor Agricultural Institute, Bogor, Indonesia. All procedures involving animals performed in the study were approved by the Institutional Animal Care and Use Committee for Primate Animal Studies of Bogor Agricultural Institute from a previous study (ACUC no. 08-B001-1R, 1 February 2010) which is further stated in Letter No.685/IT3.L1.9/TU/2019, 24 October 2019. This research was part of a bigger project where the majority of the organs of Macaca nemestrina were used.

These macaques were divided into four groups in accordance with their COH protocols, which include administration of (Gonadotropin-releasing hormone) GnRH agonist and recombinant Follicle-stimulating hormone (FSH) (r-FSH) with dosages of 30 IU, 50 IU, 70 IU (intervention groups), and no r-FSH (control group).

***Controlled ovarian hyperstimulation protocol:*** In accordance with GnRH agonist long protocol, 160 µg GnRH agonist (Suprefacts; Sanofi SA Paris, France) was administered for approximately 14 days, from the beginning of the luteal phase of the previous menstrual cycle until the day before ovulation. On the second day after menstruation, if the estradiol level reached<70 pg/mL, recombinant FSH (Gonal-F; Merck KGa Darmstadt, Germany) was injected with three different doses according to the treatment groups (30, 50, and 70 IU) per day for 10 or 12 days until estradiol secretion's peak. Then, to stimulate ovulation, HCG (pregnyl; Merck KGa) was administrated at a dosage of 3200 IU (equivalent to 10,000 IU for humans). The luteal phase was determined by measuring serial progesterone levels starting on post-ovulation day. Endometrial tissues were then retrieved on day 20-22 of the midluteal phase and embedded in paraffin blocks.

***Sample examination:*** The staining of endometrial tissues retrieved from paraffin blocks was conducted using immunohistochemistry method. Firstly, endometrial tissues of Macaca nemestrina embedded in paraffin blocks were cut with a thickness of 3,5 µm. Antigen retrieval was done in a container containing Tris Ethylenediaminetetraacetic acid (EDTA) (pH=9) which was heated in Retrieval Generation One BioGear with a temperature of 98$^{o}$C for 15 minutes. Incubation with leptin monoclonal antibody was conducted in a 4$^{o}$C temperature for 24 hours (overnight). E2 serum concentrations were obtained from our previous study (9).

***Image acquisition:*** For each tissue, we measured the immunoreactivity of three endometrial components: glandular, stromal, and luminal components. Images were obtained using a camera and IndoMicroView software. Images were taken using x40 objective, with x1, 5 magnifications.

***Data extraction:*** Endometrial component images were exported from IndoMicroView software as TIF images. Immunoreactivity to leptin is divided into four groups based on the number of positive cells: negative, low positive, positive, and high positive. The percentage of each cell group was quantified automatically with IHC Profiler plugin of ImageJ. Each component immunoreactivity was measured on

four different slide areas, randomly picked by the authors. These percentages were used to score the histological score (Hscore) for each slide, with a formula as follows (pi is the percentage of cells with *i* stain, with *i* being immunoreactivity).

$$Histological\ Score = \sum pi\ x\ i$$

***Statistical analysis:*** Data were presented as mean. Comparison of Hscore between control and intervention groups of each compartment was analyzed parametrically with Analysis of variance (ANOVA) and nonparametrically with the Kruskal-Wallis test, using SPSS release 22. Correlation between groups of E2 concentration and leptin expression was further analyzed with the Kruskal-Wallis test. Statistical decision was done using 5% significance level (p=0.05).

## Results

**Immunolocalization of leptin expression in Macaca nemestrina endometrium**

The expression of leptin in the mid-luteal endometrium phase of Macaca nemestrina was found in the cytoplasm of glandular epithelial cells, stromal cells, and luminal epithelial cells, with the level being consistently higher in the latter (Figure 1). These results were in line with leptin expression in the endometrium with natural cycle (6).

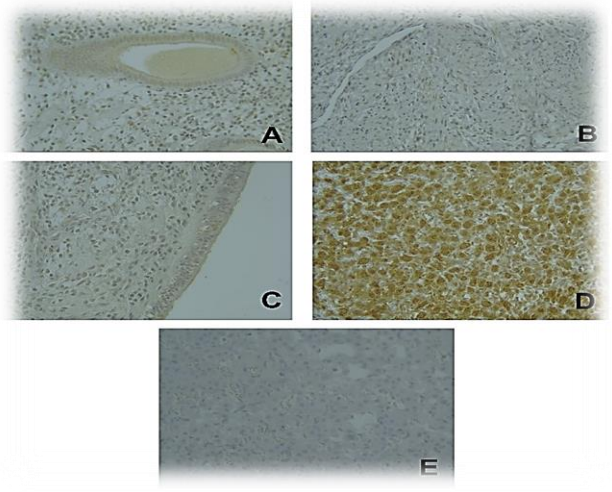


**Figure 1:** Immunohistochemical localization of leptin expression in Macaca nemestrina endometrium. Localization of leptin expression in cytoplasmic glandular epithelium cells (A), stromal cells (B), and luminal epithelial cells (C) in 400x magnification. Positive control (D) and negative control (E) are also shown.

**Leptin expression between different stimulation protocols**

Our result found no significant differences in leptin expression in the endometrial tissue between different stimulation protocols (Figure 2).

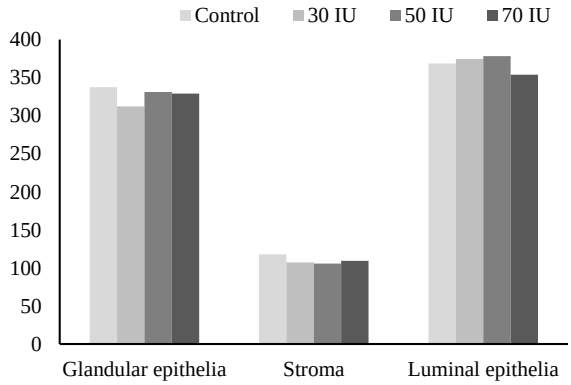


**Figure 2:** Graphical representation of leptin expression (quantified as Hscore). No significant differences in leptin expression were found between different stimulation protocols.

To illustrate the effect of estradiol levels on endometrial leptin expression, based on receiver operating characteristic (ROC) curve analysis on the leptin and estradiol data, we divided the concentration levels into two groups: E2<1000 pg/mL and E2≥1000 pg/mL. We observed a correlation between E2 concentration to stromal leptin expression (p=0.043). No significant differences were found in glandular (p=0.70) and luminal epithelial (p=0.405) leptin expression (Figure 3).

## Discussion

Leptin is known to be crucial in determining endometrial receptivity. Several leptin systems associated with this association have been proposed, including γ-Epithelial sodium channel (γ-ENaC) regulation in luminal epithelial cells (5), endometrial thickness alteration through leptin activities in luminal and glandular epithelia (10), and decidualization and mother-embryo communication inhibition in stromal components of the tissue (11). Therefore, alterations of endometrial leptin expression levels after controlled ovarian hyperstimulation (COH) are postulated to cause decreased endometrial receptivity and thus, IVF success rate.

Several studies have shown evidence for the causation. Higher levels of leptin in serum (12) and follicular fluid (6, 7, 13) are found to reduce

pregnancy rate within women undergoing IVF. Our findings, however, observe no significant differences in endometrial leptin expression between different COH protocols.

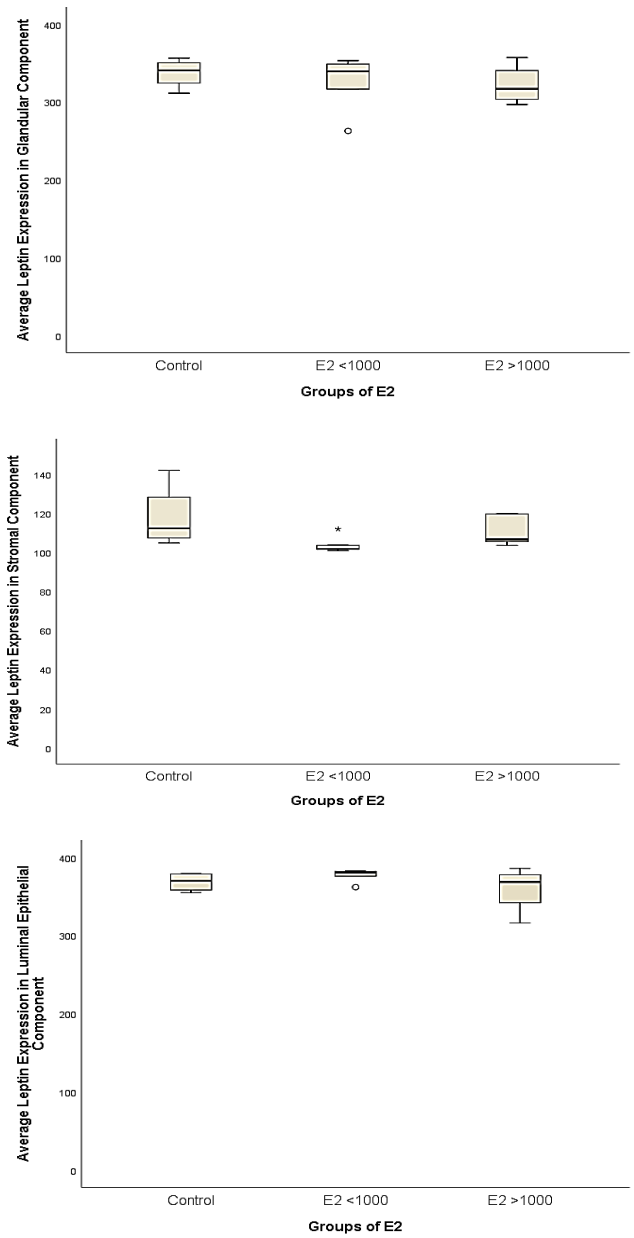


**Figure 3.** Average leptin expression within three E2 concentration groups: control, E2<1000, and E2>1000 in three endometrial tissue components: (a) glandular component; (b) stromal component; and (c) luminal epithelial component. Significant differences in leptin expression are shown in the stromal component.

The regulator of leptin expression in endometrium remains somewhat controversial. Several studies show that estradiol increases serum leptin levels within normal menstrual cycles. Another study on human endometrial tissue within different menstrual phases also supports this conclusion (14). Several studies, contradict these findings: no significant correlations was seen between serum levels of both hormones during IVF cycles, and hormone replacement therapy (HRT) of estradiol in postmenopausal women with leptin concentration (13, 15). The result of this study shows that leptin expression is correlated with higher E2 concentration (>1000 pg/mL), thus it can be postulated that estradiol acts upon endometrial leptin expression, which then exerts its effect on endometrial receptivity. In a previous study, estradiol was also found to be correlated with the expression of leptin receptor (Ob-R), suggesting its large influence on the leptin system in the reproductive organs (16).

Our study has several limitations: small sample size, limitation on standardizing the duration of each menstrual cycle phase. We also did not measure the ovarian reserve of the subjects. These limitations may affect the result of our study since they are known to affect the hormonal profile of endometrial tissues.

## Conclusion

Serum estradiol concentration is found to be correlated with leptin expression in Macaca nemestrina, suggesting a mechanism of decreasing endometrial receptivity among women undergoing controlled ovarian hyperstimulation.

## Conflict of Interests

Authors have no conflict of interests.

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