Original Research Paper

FSH Receptor and Ki-67 Protein Expressions of Endometrial Macaque in Mid-Luteal Phase after Exposure to Various Doses of Recombinant FSH and Fixed-Dose of GnRH Agonist

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Abstract: There are many advances in Assisted Reproductive Technologies (ARTs). However, implantation rates are still low. The embryo implantation is a mutual interaction between the blastocyst and endometrium. This study aimed to investigate the endometrial cell proliferation stimulated by recombinant Follicle-Stimulating Hormone (rFSH) in various doses by looking at FSH-Receptor (FSHR) and Ki-67 protein expressions. The studied samples were Stored Biological Materials (SBMs) from the endometrial tissue of *Macaca nemestrina*. All sample was obtained from fifteen macaques, divided into four groups, which three groups were treated with rFSH and given fixed-dose (160 µg/day) of Gonadotropin-Releasing Hormone (GnRH) agonists and one control group as normal group. The three treated macaque groups were stimulated with 30, 50 and 70 IU doses of rFSH. There was no significant difference in various doses of rFSH towards FSHR and Ki-67 expressions in endometrial tissue of *M. nemestrina*. Therefore, this result did not significantly correlate FSHR and Ki-67 expressions in the endometrial tissue of *M. nemestrina* after rFSH administration. Moreover, the highest rFSH dose did not reduce the FSHR and Ki-67 expressions and there was no correlation between FSHR and Ki-67 expressions. It can be concluded that macaque endometrial cells were receptive to the given dosages of rFSH.

Keywords: FSHR, rFSH, Endometrial Tissue, Proliferation Marker, Macaque

Introduction

Implantation failure is still one of the main factors that limit the success of *in vitro* Fertilization (IVF) (Simon and Laufer, 2012). Embryo implantation is a complex process that requires proper crosstalk between the embryo and endometrium (Cross *et al*., 1994; Massimiani *et al*., 2020). The IVF program success rate is still low at around 5-36% (Al-Inany *et al*., 2007; Fauser, 2019). Two main factors play a role in the success of IVF, including oocyte quality and endometrial receptivity when receiving blastocyst implantation in the endometrium (Simon *et al*., 2000; Sakiner *et al*., 2018).

Controlled Ovarian Hyperstimulation (COH) is one of the procedures for giving ovarian stimulator regimens widely used to treat patients taking IVF (Gallos *et al*., 2017; Jungheim *et al*., 2015). COH procedure aims to stimulate the growth and maturation of many follicles simultaneously (Jungheim *et al*., 2015). This procedure involves administering a drug such as Gonadotropin-Releasing Hormone (GnRH) agonist and recombinant Follicle-Stimulating Hormone (rFSH) (Macklon *et al*., 2006). The maximum number of oocytes obtained is an attempt to compensate for biological limitations and laboratory errors that may occur at the next IVF stage

(Fauser *et al.*, 2005). However, the use of gonadotropins in COH procedures can negatively affect the endometrial maturation of the implantation period (Kolibianakis *et al.*, 2002; Sahar *et al.*, 2019). It happens because gonadotropins increase progesterone and estradiol hormones in the blood serum (Lai *et al.*, 2009).

The administration of high-dose of rFSH during assisted reproductive procedures has a good impact on follicular growth (Marci *et al.*, 2013). However, the effects on endometrial cell proliferation and receptivity have not been explained. Our previous study revealed that after a fixed-dose administration of GnRH agonist and various rFSH doses, 60% of the implantation window was closed (Sahar *et al.*, 2019). However, it is currently unknown the impact of high-dose of rFSH administration on Follicle-Stimulating Hormone Receptor (FSHR) and Ki-67 protein expression on endometrial cell proliferation, which is one of the important determinants of implantation window. The purpose of this study was to analyze the development of *Macaca nemestrina* endometrium after COH by assessing the expression of cell proliferation markers and their correlation with the expression of FSHR and Ki-67 protein.

Materials and Methods

Animal

The animal models used in this study were *Macaca nemestrina* (Southern pig-tailed macaques). There are 15 female macaques of reproductive age (8-10 years, body mass of 10-15 kg) with a history of producing offspring. The number of *M. nemestrina* samples was limited due to ethical concerns (Ethical letter number: 1441/UN2.F1/ETIK/XII/2018).

The Procedure of Controlled Ovarium Hyperstimulation (COH)

These macaques were divided into five groups according to their COH protocols, including a group of GnRH agonist administration with a dose of 160 μg/day; groups of rFSH with dosages of 30, 50, 70 and a control group. GnRH agonist and rFSH were given once a day for 14 days and 12 days during the mid-luteal phase. Ovulation stimulation was conducted by administering human Chorionic Gonadotropin (hCG), which was only authorized if the macaques menstrual and ovarian cycles were successfully stabilized by the COH protocol as depicted in Fig. 1. All macaque was sacrificed by anesthetizing procedure with ketamine at a dose of 0.1 mL/kg body weight on day 20-22 of the mid-luteal phase and all endometrial tissue was collected.

Tissue dissection followed a necropsy protocol, in which the uterus was rinsed with Phosphate Buffer (PBS) and a portion of tissue was incubated in a 10% formalin solution and embedded in paraffin blocks for further analysis.

Immunohistochemical (IHC) Preparation for FSH Receptor and Ki-67 Protein

The tissue in paraffin blocks was cut with a thickness of 3.5 μm. The tissue was affixed to the glass object, which was coated with aminopropyl triethoxysilane (Apex). The tissue on the object-glass was initialized in xylene and washed with the multilevel alcohol series (70, 80, 90 and 96% ethanol, respectively), followed by washing using running water. After washing, the slide was dipped into a methanol solution containing 0.5% of H_2O_2 for 30 min and washed with running water.
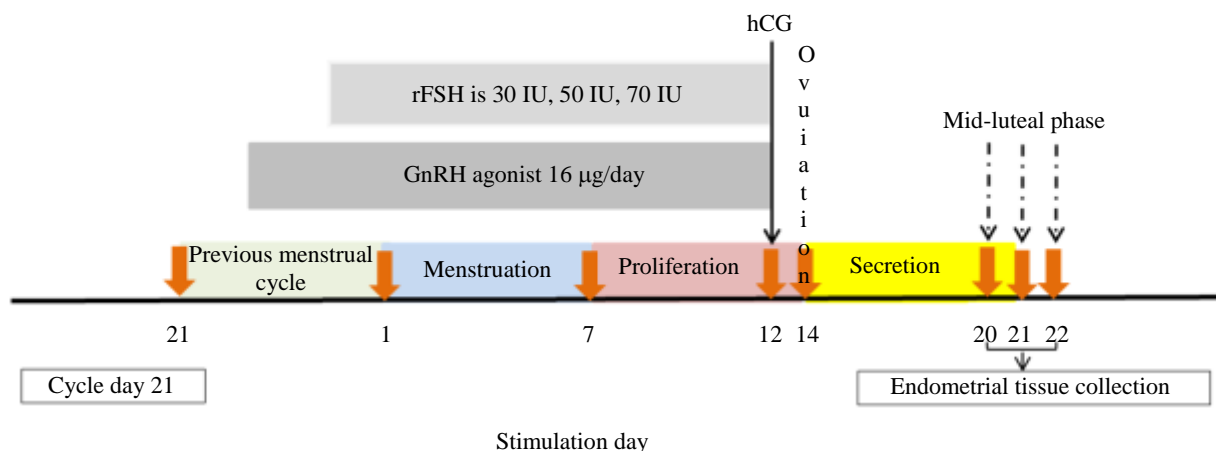

Fig. 1: The procedure of Controlled Ovarium Hyperstimulation (COH)

The appearance of tissue antigens was done by heating the slide in sodium citrate buffer (10 mmol/L, pH 6.0) for 20 min at 98°C. Endogenic peroxidase was inhibited by using 3% hydrogen peroxidase (in 50% methanol or 50% water) for 5 min, then incubated at room temperature with 5% blocking serum in a fume hood for 30 min. The slides were incubated with Ki-67 mouse monoclonal antibodies with a 100 µg/ml concentration at 1:200 dilution at 4°C overnight. Likewise FSHR, slides were incubated with FSHR monoclonal antibodies from mice (clone code: FSHR/1400) with concentrations of 1-2 µg/mL for 30 min. The next day, the slide was added with a second antibody and streptavidin-biotin-peroxidase complex. The chromogen Diaminobenzidine (DAB; Sigma Chemical) was added and stained with hematoxylin and then it was washed in running water and covered with entellan.

Assessment of Endometrial Histology

Assessment of Capping Protein Interaction (CPI) was carried out under a light microscope (binocular) with a magnification of 400 times. The assessment of the color intensity of each sample was carried out at five fields of view. The CPI assessment of the FSHR was based on the intensity of brown color in the epithelial cells of the endometrium and it was done manually by two expert observers. The cell counter plugins of Image J were used to count positive cells. Each field of view has a minimum of 100 cells. The result of two observers then analyzed using a t-test based on data distribution. No significance

on the intensity resulted. Thus it could be calculated as a mean and used as H_score. Similar to the CPI assessment of Ki-67, it was done using Image J software with IHC profiler plugin, a plugin to analyze the CPI output results using computerized color deconvolution color intensity. Thus, it processed the intensity values automatically for each cell.

The interpretation of that protein was made semi-quantitatively using the formula of H_score that was calculated using the following equation: H_score = ΣPi (i +1), where Pi is the percentage of stained cells, varying from 0 to 100% and it is the intensity of staining with a score of 1, 2 and, 3 is equal to weak, moderate and strong, respectively (Sahar *et al.*, 2019a).

Data Analysis

Statistical analysis was performed using SPSS 25.0 (IBM Corporation). Data were analyzed parametrically by ANOVA and non-parametrically by Kruskal–Wallis, where the value of $p < 0.05$ was considered as statistically significant.

Results

The expression of FSHR in the endometrium mid-luteal phase of *Macaca nemestrina* was detected in the cytoplasm and membrane cell of the stromal cells; and cytoplasm of glandular epithelial cells (Fig. 2). Strong immunoreactivity was seen in the cytoplasm of glandular epithelial cells.
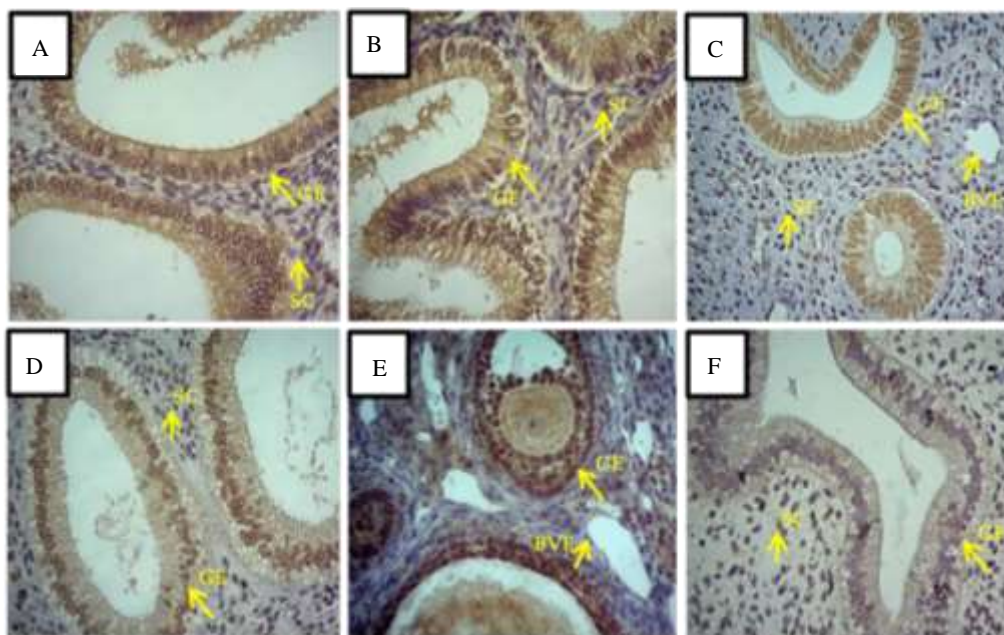

Fig. 2: Immunohistochemical staining of FSHR at the GE cytoplasm of the *Macaca nemestrina* endometrium. (A) Control group; (B) rFSH treatment at 30 IU; (C) rFSH treatment at 50 IU; (D) rFSH treatment at 70 IU; (E) Positive control of MCF7 breast cancer cells showed a positive response to anti-FSHR; and (F) Negative control of endothelial cell showed a negative response to anti-FSHR. The optical magnification was 400x. BVE: Blood Vessel Endothelium; GE: Glandular Epithelium; SC: Stromal Cell
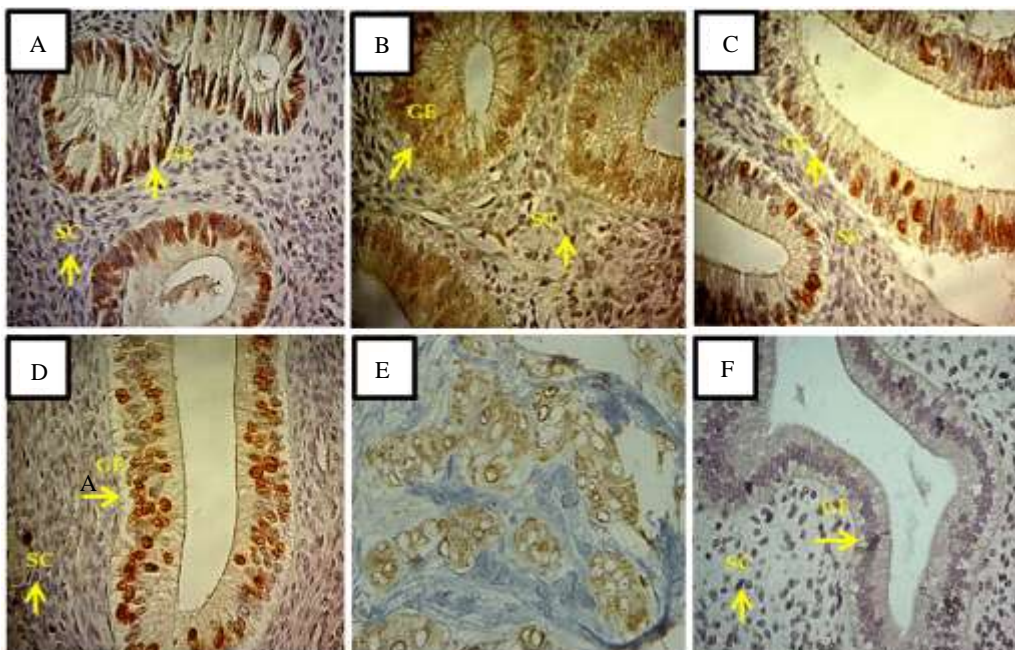
Fig. 3: Immunohistochemical staining of Ki-67 at the GE nucleus of the _Macaca nemestrina_ endometrium. (A) Control group; (B) rFSH treatment at 30 IU; (C) rFSH treatment at 50 IU; (D) rFSH treatment at 70 IU; (E) Positive control of MCF7 breast cancer cells showed a positive response to anti-Ki-67; and (F) Negative control of endothelial cell showed a negative response to anti-Ki-67. The optical magnification was 400x. GE: Glandular Epithelium; SC: Stromal Cell
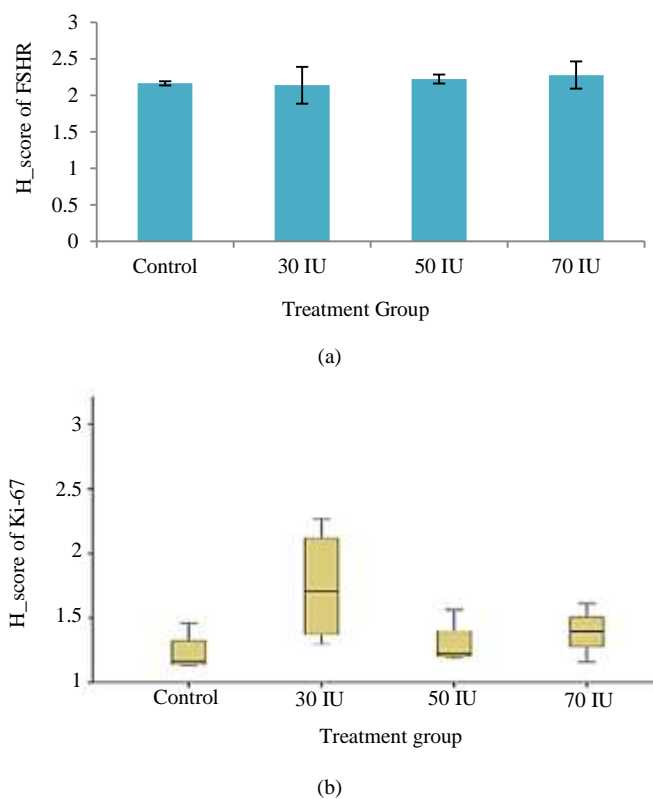

(a)

(b)

Fig. 4: H_score of endometrial protein expressions. (a) H_score value ± SD of FSHR expressions between groups; (b) H_score value ± SD of Ki-67 expressions between groups
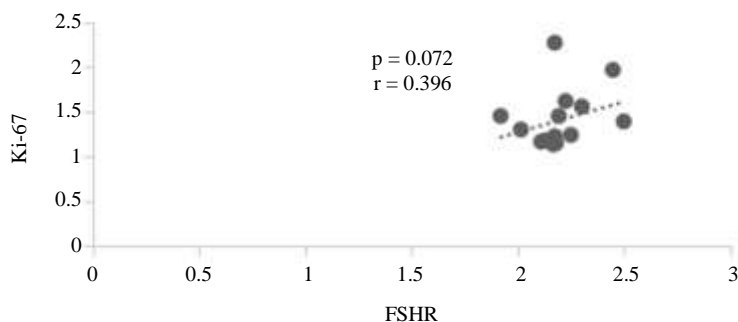
Fig. 5: Correlation between the average value of FSHR scores and Ki-67 scores

The immunolocalization of Ki-67 protein expressions in the mid-luteal phase of *Macaca nemestrina* endometrium was detected in the nucleus of the glandular epithelial cells and stromal cells (Fig. 3). However, strong immunoreactivity was found in the nucleus of glandular epithelial cells.

The average FSHR expressions amongst three groups in a different dose of rFSH treatments were found as dose-dependent in the stimulated cycle group compared to the normal cycle group, although the Kruskal-Wallis test showed no significant differences ($p = 0.642$) between each group, as shown in Fig. 4.

Likewise, the H_score value of Ki-67 expression in the stimulated cycle also did not show a significant difference between each group (Fig. 5).

The mean test result between doses of FSHR and Ki-67 protein expressions was shown no significantly different ($p = 0.961$) from the expression of Ki-67 in each group (Fig. 5). The correlation test between FSHR expression and Ki-67 protein expression showed no significant difference ($p = 0.061$). Thus, there was no significant relationship between the level of FSHR expression and the decrease of Ki-67 protein expression in each group.

Discussion

This study showed no significant differences between the higher rFSH doses and the decrease in FSHR expressions. However, FSHR expression was higher at 70 IU stimulation dose than 30 and 50 IU stimulation doses and the control group. This study followed Ponikwicka et al., which performed using endometrial tissue culture presented at 100 IU/L of rFSH. It was reported that FSHR expression in the endometrium had increased compared to controls (Sakiner *et al.*, 2018). Other researchers reported that FSH and other growth factors were considered to play a role in the FSHR regulation (Ponikwicka-Tyszko *et al.*, 2016; La Marca *et al.*, 2005).

The samples taken in the secretory phase of the menstrual cycle of endometrium referred to Ponikwicka-Tyszko *et al.* (2016) and La Marca *et al.* (2005), the FSH receptors were more commonly found in the secretory phase. More expression of FSH receptors in the secretory phase confirmed the assumption that FSHR is regulated by progesterone (La Marca *et al.*, 2005). In addition to the FSH hormone, the progesterone hormone is thought to regulate FSHR expression. A previous study reported that the progesterone hormone level in the secretory phase increased compared to the final follicular phase (Lawrenz and Fatemi, 2017). The progesterone increased levels in the mid-luteal phase may trigger the increasing FSHR expression (Sahar *et al.*, 2019a).

Another study reported that a low dose of rFSH at 4 IU/day in female rats could increase FSHR mRNA expression regulation, while a high dose of rFSH at 20 IU/day can deregulate FSHR gene expression in the ovary. Low-dose of FSH may increase the number of FSH binding sites in the ovary, together with an increase in FSH receptor mRNA. High-dose of FSH could reduce FSH receptor binding ability and decrease mRNA levels, emphasizing gene expression and internalizing receptors on female ovaries (Simoni *et al.*, 1997). Certain doses of FSH can increase FSHR expression, but high doses are thought to suppress FSHR expression through negative feedback as the response rate of FSH is too high. Thus, it suppresses the FSHR mRNA synthesis. In contrast, our result may be different from the previous report because the used pathway is different or can be caused by insufficient doses to provide negative feedback (LaPolt *et al.*, 1992).

The sampling was carried out a week after the last rFSH exposure. Thus it might be unclear whether FSHR expressed in the secretory phase was affected by the various rFSH dose or the endogenous FSH. The pattern of FSH hormone levels in the blood before rFSH administration (day 2), last day of administration (day 12) and sampling time (day 21) showed an increase of FSH in blood in 12-13 days, then FSH decreased during sampling time (day 21). This limitation might affect the data analysis, which showed no significant difference.

The high doses of rFSH tended to reduce Ki-67 protein expression even though there was no significant difference. FSH is known to induce aromatase activity. The aromatase transcription gene is also found in the endometrium of infertile patients (Brosens *et al.*, 2004). Therefore, FSH plays a vital role in endometrial

aromatase synthesis by inducing local endometrial estrogen. Local estrogen production is thought to play an important role in endometrial remodeling. Therefore, the endometrium becomes receptive during the implantation period (Fujiwara, 2009; Van Mourik *et al.*, 2009).

In line with previous studies, Zhang *et al.* (2015) reported using rodent models that low rFSH doses (0.15 IU) could reduce the size and weight of mice uterus. Other *in-vitro* experiments on endometrial cells were carried out using different rFSH doses of 50, 100 and 150 IU, which can significantly inhibit proliferation by decreasing c-Jun expression regulation and increasing caspase regulation 3/8/9 expression to activate cell apoptosis (Zhang *et al.*, 2015). Ku *et al.* (2002) performed that FSH might suppress proliferation in endometrial stromal cells.

This study revealed that FSHR expression did not significantly affect Ki-67 expression. These results suggest that FSHR may not be involved in the regulation of proliferative suppression by high doses of rFSH stimulation.

Conclusion

It was found that there was no correlation significantly between FSHR expression and Ki-67 expressions in endometrial *Macaca nemestrina*. Higher rFSH doses did not reduce FSHR and Ki-67 expressions. Thus, macaque endometrial cells were receptive to the given dosages of rFSH.

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Author Contributions

Nurhuda Sahar and Rosalina Thuffi contributed to the conceptualization, methodology, validation, supervision, writing-original draft preparation, review and editing.

Ponco Birowo, Andhea Raden Muharram, Zulhulaifah Wa Ode, Rijal Subahar, Fatmawaty Yazid, Supri Irianti Handayani and Vivitri Dewi Prasasty: Conduted formal analysis, data curation, writing-original draft preparation, review and editing.

Ethics

The Research Ethics Committee approved all the experimental protocols with macaque models of Faculty of Medicine Universitas Indonesia (1441/UN2.F1/ETIK/XII/2018).

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