Endometrial αvβ3 Integrin Expression in *Macaca nemestrina* Endometrium after Gonadotrophin Administration for Controlled Ovarian Hyperstimulation

Nurhuda Sahar[1]*, Ichramsyah A. Rachman[2], R Muharram[2], Nuryati Chairani Siregar[3], Kusmardi Kusmardi[3], Ninik Mudjihartini[4], Andhea Debby Pradhita[5], Rosalina Thuffi[5]

1. Department of Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
2. Department of Obstetri Gynaecology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
3. Department of Pathology Anatomy, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
4. Department of Biochemistry, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
5. Biomedical Science Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

Abstract

Controlled ovarian hyperstimulation (COH) has a detrimental effect on endometrial reception during the implantation period. Here, this effect is estimated based on changes in αvβ3 integrin expression as an indicator of endometrial reception. Four groups of four *Macaca nemestrina* were randomly assigned to control and three treatment groups. The treatment group received gonadotrophin recombinant follicle stimulating hormone (rFSH) at 30, 50 and 70IU through a long protocol method. Blood collected before ovulation, on the day of hCG administration. Endometrial extraction is carried out in the midluteal phase, at 8 - 10 days after the highest peak of estradiol. Integrin expression of αvβ3 was assessed by immunihistochemistry and steroid hormone by the enzyme linked immunosorbent assay (Elisa) test. The result found that there was no significant difference between the control group αvβ3 integrin expression and the treatment group (P = 0.107). No significant relationship also found between the expression of αvβ3 integrins and estradiol and progesterone levels on the day of hCG administration. Compared with progesterone levels on the day of hCG secretion, the expression of αvβ3 integrin decreased with progesterone concentration > 6 ng / mL.

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Introduction

Controlled ovarian hyperstimulation (COH) is a standard procedure for in vitro fertilization (IVF), in which a combination of gonadotropin and gonadotropin releasing hormones are injected to get many mature oocytes at the same time. IVF with husband's sperm is expected to get an embryo for potential implantation. However, at present there are no indicators that can predict the success of implantation after transfered of good quality embryo to the uterus.

Secretion of steroid hormones (estrogen and progesterone) occurs during follicle growth and development. Many studies have concluded that high estradiol and progesterone concentrations on the day of hCG administration have a negative effect on endometrial reception.[1] The effect is lack of synchronization between the developing endometrium and the phase of the menstrual cycle.[2,3] There has been no suitable method for assessing endometrial reception during the implantation period. The histological dating system first proposed by Noyet et al. 1950, also less accurate; because the variation between observers is quite high.[4]

The appropriate endometrial stage for embryonic implantation is characterized by morphological changes and expression of various proteins. The expression of this protein can be detected in the luteal phase and has been proposed as a window marker implantion.[5] Various integrins have been expressed in the luminal epithelium and endometrial gland.[6] Each integrin consists of two different subunits, namely α and β which are cyclic expressed in the endometrium during the menstrual cycle. The

***Corresponding author:**
Nurhuda
Department of Biology,
Faculty of Medicine, Universitas Indonesia,
Jakarta, Indonesia.
E-mail: nurhudasahar@yahoo.com

expression of this protein increases in the midluteal phase according to progesterone secretion, along with the time of implantation.[7-9]

The αv β3 integrin function as a potential receptor for embryo during implantation.[10,11] Impantation failure is characteristic by decreased expression of the αvβ3 integrin.[12] The expression of the αvβ3 integrin of the endometrium is temporal and related to the stage of development of the endoemtrium. The expression of the αvβ3 integrin is significantly reduced in the endometrium of infertile women[13,14], recurrent abortion, hydrosalping and unexplained infertility.[15-17]

αvβ3 integrins are cellular factors that have been widely studied as markers of human endometrial reception.[18-20] The expression of integrin αvβ3 occurs at the beginning of the secretion phase and increases the intermediate secretion phase together with increased progesterone secretion [8]. Some studies found significant differences in the pattern of αvβ3 integrin expression between natural cycles and stimulation.[21-23] The effects of ovarian stimulation on the endometrium in the expression of integrins αvβ3 have been extensively studied, but the results are different. Some studies report that in patients undergoing assisted reproduction or ovarian stimulation procedures, the expression of αvβ3 integrins is reduced, interfering with the embryo reception process, although other researchers have conflicting results.[24-27]

The porpuse of this study was to explain changes in the in αvβ3 integrin expression in the endometrium *Macaca nemestrina* after administration ovarian stimulator at 30 -70 IU/day for 10-12 day and to determine the relationship between steroid hormone level on the day of hCG administration and in αvβ3 integrin expression.

Methods

Animal

The animal used in this experimental study were female *Macaca nemestrina* at reproductive, age 8 - 10 years, with body weight of 5 - 8 kg that had already given birth. The animal was obtained from the primates animal study center of the Bogor agricultural institute Bogor, Indonesia. The study protocol was approved by the institutional animal care and use committee for primate animal studies of Bogor agricultural Institute.

The animal selected for use in this study were tattooed with an identification number in the groin area, housed in individual cages of stainless material in a dedicate 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.

COH procedure

For the COH procedure, a combination of gonadotropin was administered with a long GnRH protocol, using one of the three following regimens; gonadotropin /recombinant Folicle stimulating hormone (Gonal-F; Merck KGa Darmstadt, Germany), 2; GnRH agonist (Suprefacts; Sanofi SA Paris, France; 3; hCG (pregnyl; Merck KgaA). The GnRH agonist was administered at a dose of 160 ug to beginning in the luteal phase in the middle of the previous menstrual cycle and continued until the day before ovulation (approximately 14 day). After obtaining estradiol hormone levels of < 70 pg/mL on the second day of menstruation, administration was combained with rFSH at doses 30, 50 and 70 IU for the three treatment groups. Recombinant FSH was injected on the second day after menstruation at dose according to the treatment group each day for 10-12 day until estradiol secretion's peak. Furthermore, hCG was administrated at a dosage of 10 000 IU or equivalent to 3200 IU. The luteal phase determined by measuring serial progesterone level starting on postovulation day.

Collection of endometrial tissue sample

The uterus each animal was collected 9-10 days after the peak of estradiol secretion in normal menstrual cycle and the stimulated menstrual cycle groups. Before surgery, each animal was anesthetized with ketamine at a dose of 0.1 ml/kg body weight. At necropsy the uterus was rinsed with a phosphate buffer, and portion of the tissue was incubated in 10% formalin solution and then embedded in parafin.

Immnuhistochemical analysis of αvβ3 integrin expression

The parafin embedded tissue were cut into slices at a thickness of 0.4-0.5 um, fixed to a glass slide coated with 3-aminopropyl triethoxy silane; Sigma Alderich corporation, St Louis, MO, USA), air dried and then defaraffinized in xylen;

followed by series of decreasing alcohole concentrations. The slides were washed under running water, followed by methanol containing 0.5% H2O2 for 30 min and then washed again under running water. Antigene retrieval performed using 0.1 M trisodium citrate buffer fluid in a microwave at hight power for 3 min, at low power for 30 min, then cooled and washed ffered with phosphate buffer saline (PBS; pH 7.4) Afterward, 3% of normal horse serum (vector laboratories, Burlingame, CA, USA) was added and the slide were incubated for 20 min at room temperature. After washing, subsequent preparations were incubated with anti αvβ3 integrin (Clone LM 609; dilution 1:50, chemicon international, inc, Temecula CA, USA) at room temperature for 30 min.

Afterward, the preparations were washed with PBS, incubated with the appropriate secondary antibodies (Treki universal products) for 30 min, rinsed with PBS and subsequently incubated with horse radish proxidase labeled stertavidin (biocare medical LLC, Pacheco, CA, USA) for 30 min, before they were washed again. Nex, the chromogen 3,3 diamnobenzildine (Biocare Medical was added and the slide were allowed to stand for 1 min, then was under running water. After washing, the slides were stained with hematoxylin and covered with entelan® cover slides (EMD Milipore Corporation, Billerica, MA USA) and photographed under a light microscope at magnification x 100. The stained and unstained cells were enumerated using Image J software. Human luteal phase endometrial tissue was used as a positive control and endometrial tissue that had not reacted with the being examined was used as a negative control.

Immunohistochemical assessment

The stained slide were observer under a light microscope at magnification x 400. Percentage of stained membrane cells was assessed based on the intensity of brown coloring. The staining intensity of the endometrial components was evaluated based on a four-point semiquantitative scoring system, as follow: (-) = no staining, (+) = weak or focal staining, (++) moderate staining and (+++) strong staining. The intensity of brown color that appeared on stock was also assigned a score. Strong intensity was given a positive score of + 3; medium intensity +2; weak intensity +1 and value 0 was assigned for no staining (Table 1). The percentage of stained cells in five field of view was determined. The histology score was calculated according to following formula: H-score ∑pi (i+1). Where pi is percentage of stained cells (range 0% - 100%), i is the coloring intensity (range 0-3) and 1 is a correction factor for optical density. The value for each preparation is the summation of the average value assigned for positive staining and color intensity.

Statistical analysis

All quantitative data were analyzed using SPSS 17. The concentration of αvβ3 integrin 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 P to αvβ3 integrin was analyzed using Spearman. $P \leq 0,05$ was considered significant.

Results

Changes in levels steroid hormone and integrin αvβ3 after COH

The means estradiol and progesterone levels after hCG administration in the control and stimulated groups are presented in Table 2. According to the analysis of variance (ANOVA) results, there were no significant differences in estradiol and progesterone levels between the control and treatment groups ($P > 0.05$).

The αvβ3 integrin expression level of the *Macaca nemestrina* endometrium determined based on the surface of the epithelial cells of the endometrial gland. All 15 immunohistochemically examined endometrial tissue sample were stained to varying intensities. The average staining intensity appeared stronger in the control group than the treatment groups (Figure 1). Using H-score formula ∑pi (i+1), the average H-score of αvβ3 integrin expression in the control group and the 30,50 and 70 IU treatment groups were 2.30, 2.09, 1.95 and, 1.97, respectively (Table 1 and Figure 1). Based on ANOVA results, there was no statisctically significant difference between the control and treatment groups ($P > 0.05$).

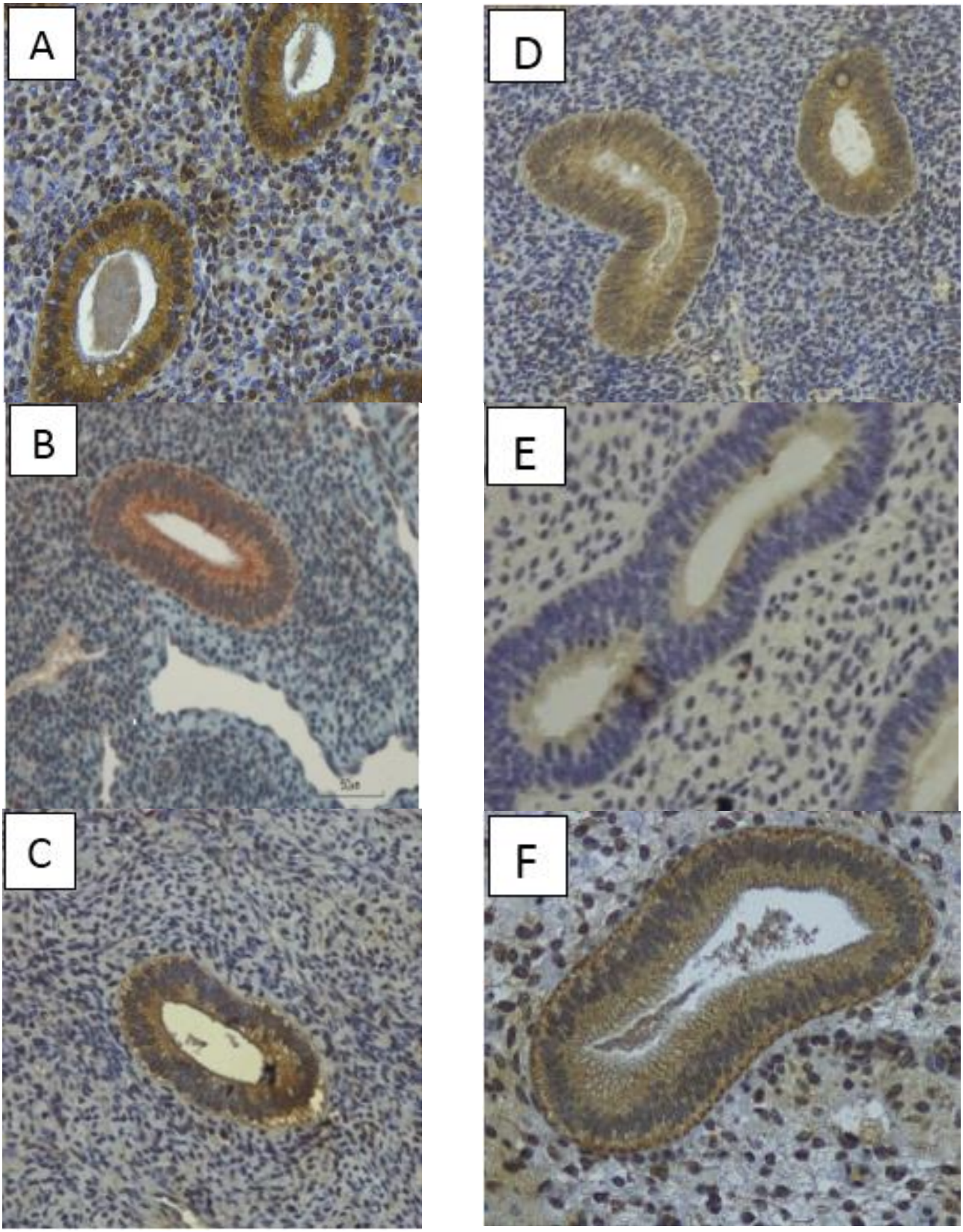
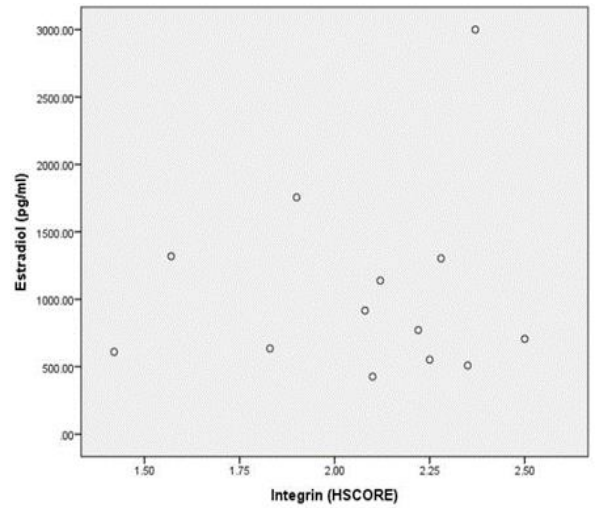


Figure 1. Immunohistochemical staining of αvβ3 integrin of the *Macaca nemestrina* endometrium. (A) control group, (B). The treatment wiyh 30 IU, (C) treatment with 50 IU, (D) treatment with 70 IU, (E) negative control, and (F). positive control. Original magnification 400x.

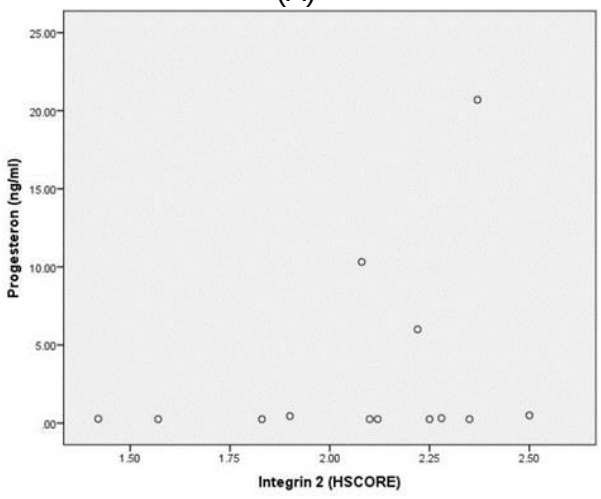
Positive value	Criteria
+3	Strong
+2	Moderate
+1	Weak
0	Negative

Table 1. Staining Intencity.

Correlation between estradiol and progesterone level during the day of hCG administration with αvβ3 integrin expression level of the *Macaca nemestrina* endometrium



(A)



(B)

Figure 2. Graph correlation of estradiol (A) and progesterone (B) level on the day of hCG administration and αvβ3 integrin expression.

In this study, the relationship between estradiol and progesterone secretion level on the day hCG administration with αvβ3 integrin expression were analyzed. The goal was to determine wether αvβ3 integrin expression was affected by estradiol and progesterone level. Figure 2, shown a correlation graph of αvβ3 integrin expression versus estradiol and progesterone levels. According to the Pearson correlation test result there was no significant association between estradiol and progesterone level with αvβ3 integrin ($P > 0.05$), as higher level of estradiol and progesterone secretion were not followed by increase in αvβ3 integrin expression.

This study analyzes the rate of increase in early follicular phase progesterone levels with αvβ3 integrin expression, as compared with a progesterone level of < 1 ng/ml (Table 3) although no statistical difference. The average H-score of αvβ3 integrin expression at progesterone level < 1 ng/ml and > 6 ng/ml was 2.15 and 1.94.

Hormone levels andαvβ3 integrin	Normal	Menstrual cycle			P-value
		Stimulated dose 30 IU	Stimulated dose 50 IU	Stimulated dose 70 IU	
Estradiol (pg/mL)	548 ±95	874±640	1290±937	1042±194	0.364
Progesterone (ng/ml)	0.31±.24	0.89±3.56	7.80±41.5	2,16 ±6.5	0.291
αvβ3 Integrin	2.30±0.17	2.09±0.27	1.95±0.4	1.97±0.35	0.107

Table 2. Estradiol and progesterone levels following hCG administration days and H-score of αvβ3 integrin expression.

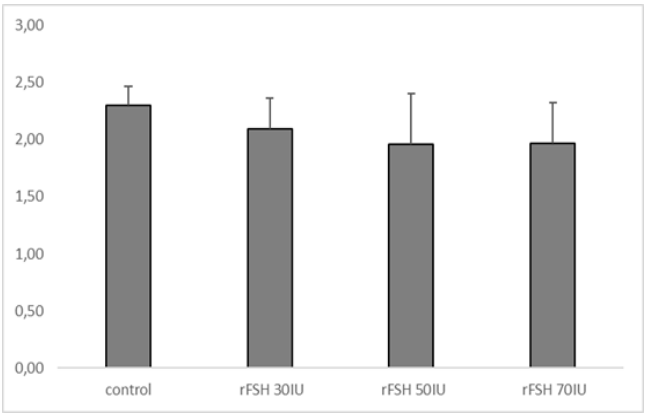


Figure 3. Histogram of the integrin αvβ3 H-score of the *Macaca nemestrina* endometrial control group and stimulated groups I, II, and III.

Progesterone	Range	H-score of αvβ3 Integrin expression
< 1 ng/ml (n=8)	0.25 -0,49 ng/ml	2.15
> 6 ng/ml (n=3)	6-20.7ng/ml	1.94

Table 3. Comparison of progesterone levels on day of hCG administration with αvβ3 integrin expression (H-score).

Discussion

The success of embryo implantation is determined by two main factors; the quality of the embryo and the maturation level of the endometrium during the implantation period. Although laboratory parameter to evaluation embryo quality have been estabilished, but there is currently no concensus or an indicator of endometrial receptivity. Many studies have reported that endometrial receptivity during the implantation period is a disrupted by COH procedure,[28-32] but the degree of disruption is difficult to assess. The αvβ3 integrin is one of the most important endometrial receptors. Expression of integrin has been correlated with the maturation stage and optimal expression occurs in the mid luteal phase at the time of implantation.[31]

In this study. We analyzed changes in the level of αvβ3 integrin expression of *Macaca nemestrina* endometrium in the immunohistochemical midsecretion phase in natural cycle group and three treatment groups administered gonadotrophin (Gonal-F), at doses of 30,50 and 70 IU/day for 10-12 days. Using the H-score formula, αvβ3 integrin expression was lower in the treatment groups than in the control group. The lower expression level of αvβ3 integrin in the stimulated groups can be associated with demage uterine reception. The important role of αvβ3 integrin as a determinant of the opening of the implantation window requires greaters study and adequate statistical power, ideally investigating more than one menstrual cycle in some women. Other investigators concluded that the expression of the αvβ3 integrin in the endometrial biopsy samples of the midsecretion phase had no correlation with infertility,[29,30] or undergoing an assisted reproduction regimen.[31]

The decrease in αvβ3 integrin expression in the stimulated menstrual cycle is probably an indirect effect of COH. Studies have shown that COH causes changes in the menstrual cycle, which progressed for two to three day before the implantation period.[3] It is known that optimal αvβ3 integrin expression occurs at 9-10 day after ovulation or in the implantation period.[18,19] Some our data show that αvβ3 integrin expression values were not optimal in the stimulated group, which may have been due to an inappropriate period of menstruation, as a result of COH. The decrease in αvβ3 integrin expression is consistent with the dosage of the stimulator administered. The dose of rFSH administration contributes to a decrease in αvβ3 integrin expression. When compared with oher report we found similarities in the indirect implantation of an impaired embryo when the developing endometrium is affected by the effects COH.[32,33]

A number of previous studies have reported on the expression of endometrial αvβ3 integrin in the stimulated cycle but have produce inconsistent results. One study showed increased αvβ3 integrin expression in women undergoing IVF followed by pregnancy,[34,18] while others showed different results. Others studies found no difference in endometrial αvβ3 integrin expression between IVF or intracytoplasmic sperm injection (ICSI),[35] or even unsuccessfull, -IVF-embryo transfer.[33] Hence, it seems at implantation failure in stimulated cycle not always associated with normal αvβ3 integrin expression, but possibly is antother factor.

Receptivity endometrial development of embryonic implantation is controlled by an adequate estrogen and progesterone response. In the follicular phase of a normal menstrual cycle, estradiol partly regulates the proliferation of endometrial cells and progesterone maintains pregnancy and regulates the monthly menstrual cycle. Gradually increased sercretion after ovulation of is optimal in the midphase of secretion. Early progesterone or early luteinization surges often occur in ovarian stimulation procedure, In the present study there was an incidence of progesteron surge of 27% (3 samples) with progesterone at > 6 to 20.7 ng/ml, while there was no concentration increase in eight samples (< 1 ng/ml).

The increased impact of the serum progesterone consequences have remained controversial for years, especially threshold values. Some studies have found no accosiation between progesterone level and pregnancy rates.[33] While others have reported negative effects on cyclical outcomes when serum progesterone levels rise.[34] When accosiated with αvβ3 integrin expression as a marker of decreased endometrial receptivity, the H-score decreased from 2.15 to 1.94. Althought the number of *Macaca nemestrina* samples was limited, our finding suggests that high progesterone level on the day hCG administration tend to have a negative effect on

endometrial receptivity and may lead to implantation failure.

Conclusion

COH caused a decrease in αvβ3 integrin expression as a sign of endometrial receptivity and negatively impacted endometrial during the implantation period. Decreased αvβ3 integrin expression occurs in progesterone level in day given hCG > 6 ng/ml.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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