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Comparison of endometrial receptivity between agonist and antagonist protocol in mice uterus

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ABSTRACT

Background: The implantation rate of IVF remains low due to the adverse effect of ovarian stimulation on endometrial receptivity. Integrin β3 is the best known marker for endometrial receptivity evaluation. In this study, the impact of antagonist and agonist protocol on the expression of integrin β_3 during implantation window was investigated.

Methods: Two ovarian stimulation protocols were used, involving FSH plus GnRH agonist and FSH plus GnRH antagonist. Uterus sample were collected 48 hr after ovarian stimulation and the expression of integrin β_3 detected by immunohistochemistry.

Results: The GnRH antagonist treated mice showed significantly lower integrin β_3 expression in endometrium during window of implantation than GnRH agonist treated mice.

Conclusions: Antagonist protocol has more adverse impact on endometrial receptivity of mice uterus than agonist protocol

Keywords: GnRH antagonist, endometrial receptivity, integrin β_3, ovarian stimulation.
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INTRODUCTION

In vitro fertilization (IVF) is the most popular method to assist pregnancy program especially among couple who difficult to pregnant. Despite the improvement in IVF tehnology and care, the implantation rate of this tehnique remain low. For example, the implantation rate in US is 29%[1] and in Europe, it is estimated to be 29,3%.[1,2] Compared with implantation in natural fertilization (50%), the rate is considered low. [3]

Investigations in recent decade show that the major cause of low implantation rate in IVF is due to high level of estrogen produced by growing follicles. Estrogen is known to affect oocyte, sperm, and even blastocysts quality. In addition, endothelial receptivity is also affected which result in difficulties of blastocysts implantation.[4-8]

Endometrial receptivity is defined as the period of endometrial maturation during which the trophectoderm of the blastocyst can attach to the endometrial epithelial cells and subsequently proceed to invade the endometrial stroma and vasculature. The transition from the non-receptive to the receptive state is determined by the expression of membrane-bound, soluble, and secretory factors that support trophoblast attachment and subsequent migration. Factors expressed during this temporal window have been exploited as biomarkers of the receptive state.[9]

Because implantation process involved cell to cell interaction, it is not surprising that the best way to evaluate endometrial receptivity is by evaluating adhesion molecules. Integrin β_3 is one of the cellular factor which has been best characterized and largely accepted as the candidate of endometrial receptivities biomarker in human and mice.[10,11,12] The expression of Integrin β_3 is known to dynamically follow menstrual cycle with peak expression at mid-secretory phase of menstrual cycle. Coincidentally, this phase is also the phase when implantation occurs. Moreover, Integrin β_3 expression is also down regulated in infertile women caused by hydrosalphing, endometriosis, and PCOS and unexplained recurrent pregnancy loss.[14-16]

In this study, we investigated the effect of different ovarian stimulation protocol (agonist protocol, or antagonist protocol) on the expression of Integrin β_3 subunit in endometrium of human IVF mimicked mouse model. Based on aforementioned evidences, we used Integrin β_3 as biomarker of endometrial receptivity. We hypothesized that different ovarian stimulatian protocol would induce different Integrin β_3 expression in endometrial tissue and, hence, reflect different endometrial receptivity.

MATERIAL AND METHODS

Animals

Animal care and usage procedures were in accordance to the institutional guidelines established by the Animal Care and Use Comitte (Pitts, 2002). Virgin 8-12 week old female CD1 (Charles River,USA) mice were housed in 12hr light and

12hr dark cycle at 25 + 0.5°C and 50-60% humidity. The mice were fed ad libitum with standard diet and water. To identify the Estrus cycle, vaginal discharge and smear samples were evaluated daily. Mice that exhibited regular 4-days cycles, 18-22 gram body weight, were randomly allocated to two groups which 18 mice in each group. First group treated with agonist protocol ovarian stimulation and the second group treated with antagonist protocol.

Ovarian Stimulation

The ovarian stimulation procedure in two different group were:

1. GnRH agonist group: GnRH agonist (suprefact) was intraperitoneally (i.p) injected at 1,5µgr/100 grBW/day from day 3-9 of estrus. Then, at 9.00 a.m of day 9, FSH (Gonal F) was i.p injected at 40IU/100 grBW followed by in i.p injection of hCG (Pregnyl) 100 IU/100 grBW at 01.00 p.m on day 10.
2. GnRH antagonist protocol: Normal saline of the same volume was injected from day 3-8 of estrus at 9 a.m. At 9 a.m of day 9, FSH (Gonal F) was i.p. injected at 40 IU/100grBW, followed by injection of GnRH antagonist (Cetrotide) 4 µg/100grBW, i.p. at 01.00 p.m. At 01.00 p.m. of day 10, hCG (Pregnyl) was i.p. injected at 100 IU/100 grBW.

Tissue Collection:

Uterus samples were collected on day 11 of estrus period, 48 hrs after OS, or 29 hrs after injection of hCG. The mice were sedated by diethyl ether and then histerectomized. The uterus were fixated by 10% formalin solution and embedded in parafin.

Immunohistochemistry Analysis

Paraffinized tissues were sliced into 0.3 µm thickness tissue sections and deparaffinized in xylol solution and ethanol 90%. Antigen retrieval was conducted by boiling tissue sectioned in 10 mmol/l citrate buffer (pH 6.0) for 20 min. For immunohistochemitry detection of Integrin β_3, goat-anti-human monoclonal antibodies was used according to the kit standard. The colour was developed with betazoid DAB, counterstain with hematoxylin Meyer and dehydrated by alkohol 70-90% and xylene solution.

Staining intensity of tissue section was evaluated and graded (< 1 weak, 1.1-2 moderate, 2.1-3 strong, 3.1-4 very strong), and was assessed by using the H-SCORE that calculated using the following equation: H-SCORE = $P_i(i+1)$. Where P_i is the percentage of stained epithelial cells (0-100%), i is the staining intensity. The positive control was day 11 of mice CD1 uterus without ovarian stimulation.

Statistical Analysis

Mann-Whitney test was used to compare the expression of endometrial Integrin β_3 in different ovarian stimulation groups. p<0,05 was considered statistically significant. All data were analized using SPSS for windows 17-version.

RESULTS

42 CD1 mice were used in this study with 21 mice for each group. However, after treatment protocol and sample processing, only 36 samples were eligible for analysis. The other 6 samples contain only adipose tissue and lymph node with no uterine tissue which possibly because of processing error.

Immunohistochemical analysis was used to evaluate the expression Integrin β3. Based on mean H-SCORE of Integrin β3 expression, it appeared that agonist group tend to had higher expression of integrin β3 (1.58±0.79) compared to antagonist group (0.45+0.27). Statistical analysis for the difference of expression score reveal that the result is statistically significant (p=0,001). The detail of the data is presented in Table 1.

Intensity staining evaluations in each group revealed convincing result. It showed that there was variety of Integrin Subunit β3 expression in agonist protocol which ranged from weak to very strong. However, there was only one sample that showed very strong Integrin Subunit β3 expression and more than half of samples (66.67%) showed moderate and strong expression as shown in Table 2.

Below (Figure 1) are several immunostaining result that represent each staining classification. It was apparent that there is gradual increase in intensity and change in color appearance from weak expression to strong expression. We consider H-Score 0.6 as weak expression, 1.5 as moderate, 2.1 as strong and 3.2 as very strong.

Table 1 Mean Hscore Agonist group and Antagonist group

	H-score Integrin subunit β3		
Group	**Mean**	**SD**	**P**
Agonist protocol	1.58	0.79	0.001
Antagonist protocol	0.45	0.27	

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Table 2 **H-score proportion of Integrin Subunit β3 in agonist group and antagonist group.**

Group	H-Score proportion of Integrin Subunit β3 (%)				Total
	Weak	Moderate	Strong	Very strong	
Agonist protocol	5 (27.78)	7 (38.89)	5 (27.78)	1 (5.56)	18 (100)
Antagonist protocol	18 (100)	0	0	0	18 (100)

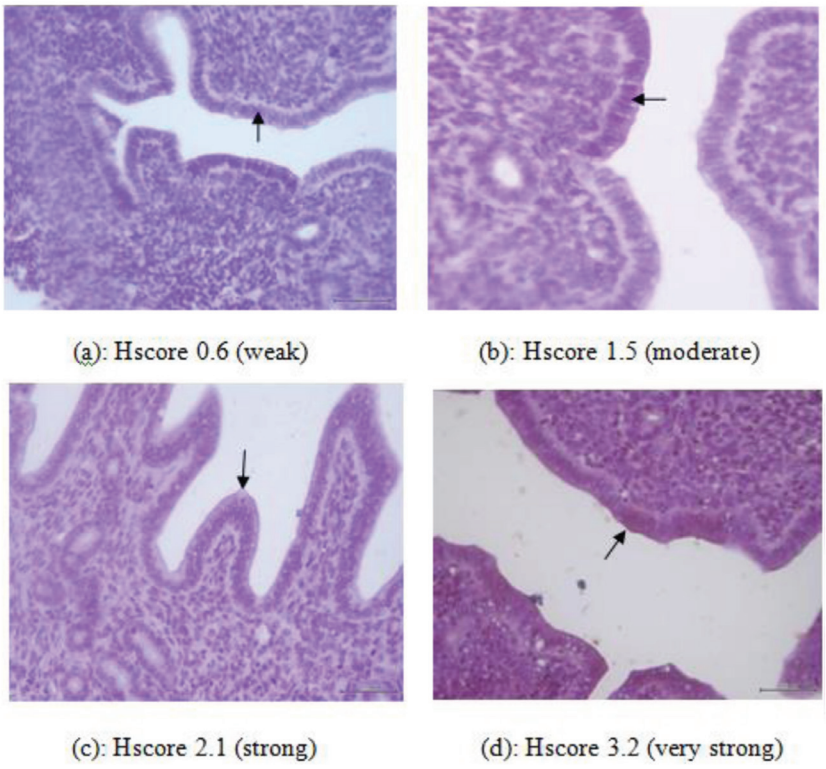

Figure 1 Classification of immunostaining intensity based on H-Score

DISCUSSION

Endometrial receptivity is an important factor for embryo implatation. In menstrual cycle, estrogen and progesteron play important role in endometrial thickening and vascular development. In the later stage of menstrual cycle, gonad hormones, particularly progesterone, induce development of secretory gland in endometrial tissue which mark the secretory phase of uterine cycle. Both hormone also modify uterine receptivity in preparation for blastocyst implantation.[17,18].

One of important marker of uterine receptivity is Integrin β_3. Several study reveal that the expression of this molecule appear in cyclical manner, in accordance with uterine cycle which made it suitable for evaluating uterine receptivity. Because of it expressional cycle, it strongly indicate that the expression is influenced by gonad hormones (estrogen and progesterone).

The aim of antagonist and agonist protocol of ovarian stimulation conducted in our study to know the expression of integrin β_3 subunit in endometrial cell during window of implantation of mice. The mean H-SCORE of agonist and antagonist group were 1.58 + 0.79 and 0.45 + 0.27 respectively (p=0.0001) which showed that integrin β_3 was significantly lower in antagonist group. The integrin β3 expression in agonist group were weak (27.78%), moderate (38.89%), strong (27.78%), and very strong (5.56%), while in the antagonist group all of the integrin β_3 expression were weak, which showed that antagonist protocol has more adverse impact on integrin β_3 expression than agonist protocol.

The adverse effect of ovarian stimulation with gonadotrophin on endometrial receptivity have been documented in previous study. The implantation rate was significantly lower in ovarian stimulated mice which fostered good and healthy embryos, compare with natural cycle control (7% vs 25%, p=0.0001).[19] Another study found significant lower in integrin β_3 (and also LIF) expression in ovarian stimulated mice. The expression integrin β_3 in antagonist group was significantly lower compare with agonist group.[20]

However, our study did not provide answer to why the implantation rate in stimulated IVF is lower than IVF with normal cycle.[1-3] It is believed that factors other than integrin and gonad hormones play significant role in modifying endometrial receptivity toward embryo. Further research is still needed to uncover the physiological process behind embryonal implantation.

CONCLUSION

In conclusion, antagonist protocol has more adverse impact to endometrial receptivity compare with agonist protocol. The low implantation rate observed in ovarian stimulation protocol in IVF clinics might partly result from the impaired endometrial receptivity through impairing integrin β_3 expression. Further study in this aspect and lowering estrogen level in ovarian stimulation are needed.

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