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An Immunohistochemical Study of Beta1 Integrin Molecules (VLA-4, VLA-5, VLA-6) in All Endometrial Compartments of Fertile and Infertile Women in Ahwaz-Iran

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Abstract: In some cases of infertility, implantation failure is due to a lack of expression of specific critical participating proteins such as cell adhesion molecules. The expression of beta 1 (β1) integrin molecules within endometrial tissue has been proposed as a marker of uterine receptivity during the implantation window. Present study was conducted to assess uterine receptivity in women with unexplained infertility using β1 integrin molecules within endometrial tissue in comparison with fertile women. This retrospective study was performed using a semiquantitative analysis on the immunohistochemical staining of β1 integrins (VLA-4, VLA-5, VLA-6) in the mid-secretory phase of endometrium. Specimens were obtained from 30 fertile women and 28 infertile patients with a history of unexplained infertility. Chi-Square test was used to compare the expression and defect of β1 integrin molecules between two groups. The results showed β1 integrin molecules were present in fertile and infertile endometrial uterine tissues with different reactivity in different compartments. VLA-5 and VLA-6 expression on endometrial compartments showed an unrelated pattern of staining in either fertile or infertile women. The majority of glandular epithelial cells and stromal cells expressed VLA-4 integrin molecules in fertile endometrium. However, the reactivity with VLA-4 reduced significantly in both glandular epithelial cells and stromal cells in infertile women ($p = 0.001$). In conclusion differences may explain causes of unexplained infertility and suggests that VLA-4 integrin molecule may contribute in uterine endometrial receptivity at the time of the implantation window which requires more investigations in benign gynecologic diseases.

Key words: Implantation, adhesion molecules, infertility, endometrial epithelial cells

INTRODUCTION

Infertility is a gynecologic disorder affecting 10B15% of women in reproductive age (Randolph, 2000). Endometrial receptivity is established during the mid-secretory phase, between Cycle Day (CD) 20 to 24 (Wilcox *et al*., 1999). Numerous cell adhesion molecules (including integrins, selectins and cadherins) are expressed by the endometrium and appear to be necessary for the successful interaction of the embryo with the endometrium (Sueoka *et al*., 1997; Achache and Revel, 2006). Recent attention has focused on the expression of integrin molecules within the endometrium and their absence in conditions related to infertility (Lessey *et al*., 1992; Donaghay and Lessey, 2007). Integrins are transmembrane glycoproteins comprising alpha and beta subunits and are expressed on many non-haematopoietic cells as well as on leukocyte cell types. Integrin-mediated cell-cell and cell-matrix interactions regulate different type of cellular activities, including inflammatory response, angiogenesis, cell migration, proliferation, differentiation and gene expression (Hynes, 1992; Abbas *et al*., 2007). They are classified into several groups according to their βsubunit.

The β1 integrin or VLA family has at least six distinct members (VLA1-6) which are expressed by a variety of cell types (Abbas *et al*., 2007). β1 integrins have been shown to undergo specific changes within the endometrium

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during different phases of the menstrual cycle (Tabibzadeh, 1992; Lessey *et al*., 1994). Due to the temporal pattern of their expression around the time of implantation and their absence in conditions related to infertility such as endometriosis, hydrosalpinges and unexplained infertility, these integrins might have an important role as a potential marker of uterine receptivity (Skrzypczak *et al*., 2001; Szymanowski *et al*., 2003; Savaris *et al*., 2007). To confirm these observations and extend investigations of endometrial expression, the present study was conducted to determine whether the endometrium of women with unexplained infertility differs in the presence of the β1 integrin subunits including VLA4, VLA5 and VLA-6 from the endometrium of normal fertile women.

MATERIALS AND METHODS

Participants: In this prospective case-control study, thirty proven fertile women (control) and 28 patients with unexplained infertility participated. Ages ranged from 20 to 41 years in both groups. All of the women were informed about the procedures and consent was obtained. Fertile specimens were obtained fresh from hysterectomies performed for nonendometrial pathology from the operating theaters. The inclusion criteria for these women included: (1) healthy and proven fertile, (2) who had regular ovulatory cycles, (3) and had not used any type of contraceptive drugs or intrauterine devices within the last 6 months. Endometrial biopsies from women with unexplained infertility were obtained by using uterine curetting from IVF Center. The inclusion criteria including infertility of a duration exceeding 1 year; normal quality sperm; normal ovulation; normal anatomical uterus and normal menstrual cycle.

All samples were obtained at the time of implantation window in secretory phase from Ahvaz Imam Khomaini Hospital during 2004-2005.

Antibodies and immunohistochemistry: Three murine monoclonal antibodies were used to label different β1 integrin markers in the all samples. All were suitable for use on frozen tissue sections. These were: monoclonal anti-human α4β1 (VLA-4), α5β1 (VLA-3) and α6β1 (VLA-3) integrins supplied from Dako Ltd.

Acetone-fixed cryostat sections from endometrial biopsies were stained immunohistochemically with three monoclonal antibodies against β1 integrin subunits. Non-specific binding was inhibited by 10 min incubation with normal rabbit serum. Tissue sections were incubated for 60 min with primary antibody and stained with streptavidin-biotin-horse radish peroxidase system (Dako LSAB kit system). The sections were then stained by DAB enzymatic produced, counterstained with Mayer's haematoxylin and finally evaluated microscopically.

Negative controls were incubated with irrelevant mouse monoclonal antibodies instead of primary antibodies. All incubations were carried out in a moist chamber at room temperature.

Statistical analysis: The reactivity of antibodies directed against integrin subunits with different compartments of the endometrium (glandular epithelium, vessels, lymphocytes, macrophages, stromal cells) was scored semi-quantitatively according to the degree of positive staining: (-) when there was no reactivity greater than that observed in the negative control; (") fewer than 5% of cells were positive; (+) 5-25% of cells were positive, (++) 25-50% of cells were positive and (+++) more than 50% of cells were positive (Klentzeris *et al*., 1993; Lessey *et al*., 1994). Data were analyzed with the program Minitab version 14.0. Two groups were compared by chi-square analysis test.

RESULTS

No reactivity was seen in all compartments of negative control slides. Immunohistochemical analysis demonstrated all the β1 integrin subunits are present in fertile and infertile uterine endometrial tissues (Table 1-3). VLA-4 expression was detected in glands, vessel, lymphocytes, stromal cells and macrophages with varies reactivity. The reaction pattern for epithelial and stromal cells was significantly different between fertile and infertile endometrial tissues ($p = 0.001$). In contrast to infertile cases, the reactivity for these compartments particularly glandular epithelial cells was positive for majority of fertile cases (Fig. 1, 2). VLA-4 reactivity was

Table 1: Reactivity of VLA-4 integrin with different compartments in secretory phase endometrium of fertile and infertile women

Endometrial compartments	-	±	+	++	+++
Glands					
Fertile	4	0	1	5	20
Infertile	15	5	0	5	3
Vessels					
Fertile	0	3	25	2	0
Infertile	0	1	22	5	0
Lymphocytes					
Fertile	0	0	24	6	0
Infertile	0	4	21	3	0
Macrophages					
Fertile	0	20	9	1	0
Infertile	2	17	10	0	0
Stromal cells					
Fertile	0	3	23	2	0
Infertile	18	2	8	0	0

-: No reactivity; ±: <5%; +: 5-25%; ++: 25-50; +++: >50%

Table 2: Reactivity of VLA-5 integrin with different compartments in secretory phase endometrium of fertile and infertile women

Endometrial compartments	-	±	+	++	+++
Glands					
Fertile	26	4	0	0	0
Infertile	27	1	0	0	0
Vessels					
Fertile	0	0	7	23	0
Infertile	0	0	9	19	0
Lymphocytes					
Fertile	0	3	20	7	0
Infertile	0	5	18	5	0
Macrophages					
Fertile	30	0	0	0	0
Infertile	28	0	0	0	0
Stromal cells					
Fertile	0	0	0	3	27
Infertile	0	0	1	4	23

-: No reactivity; ±: <5%; +: 5-25%; ++: 25-50; +++: >50%

Table 3: Reactivity of VLA-6 integrin with different compartments in secretory phase endometrium of fertile and infertile women

Endometrial compartments	-	±	+	++	+++
Glands					
Fertile	0	0	0	3	27
Infertile	0	0	1	8	19
Vessels					
Fertile	0	0	1	28	1
Infertile	0	0	3	23	2
Lymphocytes					
Fertile	30	0	0	0	0
Infertile	28	0	0	0	0
Macrophages					
Fertile	30	0	0	0	0
Infertile	28	0	0	0	0
Stromal cells					
Fertile	0	3	24	1	0
Infertile	1	4	23	0	0

-: No reactivity; ±: <5%; +: 5-25%; ++: 25-50; +++: >50%

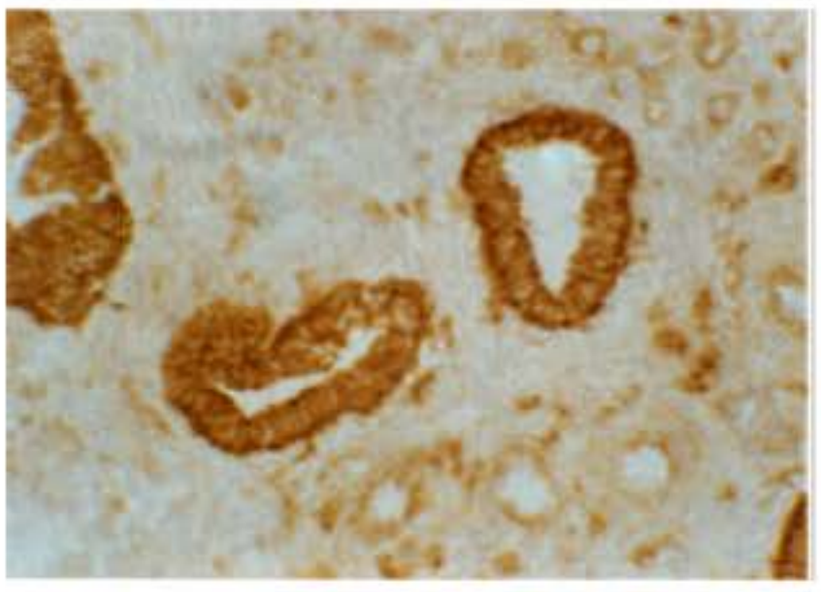

Fig. 1: Frozen section of uterine endometrium tissue of fertile women in secretory phase stained immunohistochemically with anti-VLA-4 monoclonal antibody. The maturity of glandular epithelial cells are positive for β3 integrin. Reactivity with vessels, lymphocytes, macrophages and stromal cells are also seen. Magnification x200.

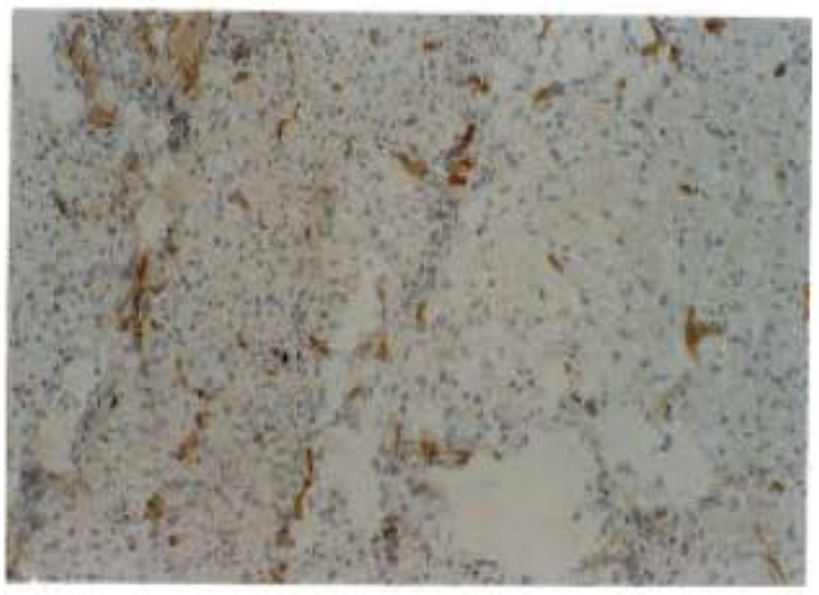

Fig. 2: Frozen section of uterine endometrium tissue of infertile women in secretory phase stained immunohistochemically with anti-VLA-4 monoclonal antibody. VLA-4 integrin reactivity with glandular epithelial cells reduce significantly in comparison with endometrium tissue of fertile women. Reactivity with macrophages, lymphocytes and vessels are also seen. Magnification x200

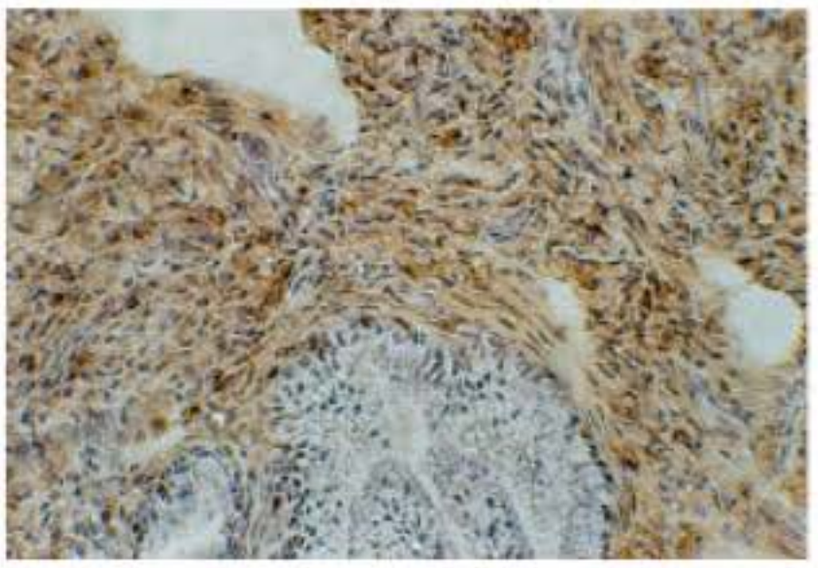

Fig. 3: Frozen section of uterine endometrium tissue of infertile women in secretory phase stained immunohistochemically with anti-VLA-5 monoclonal antibody. Note glandular epithelial cells are negative. Stromal cell reactivity is predominant. Lymphocyte positivity is also detected. Magnification x400

detected in 5-25% of lymphocytes in both fertile and infertile cases with no significant difference. The pattern of staining varied for macrophages and vessels between two groups but differences were not significant ($p = 0.84$ and 0.19, respectively).

Glandular epithelial cell reactivity for VLA-5 was negative for the majority of two groups. Stromal cell reactivity with anti-VLA-5 was predominant in all cases of fertile and infertile endometrial tissues with no significant difference (Fig. 3). Less than 50% of vessels expressed

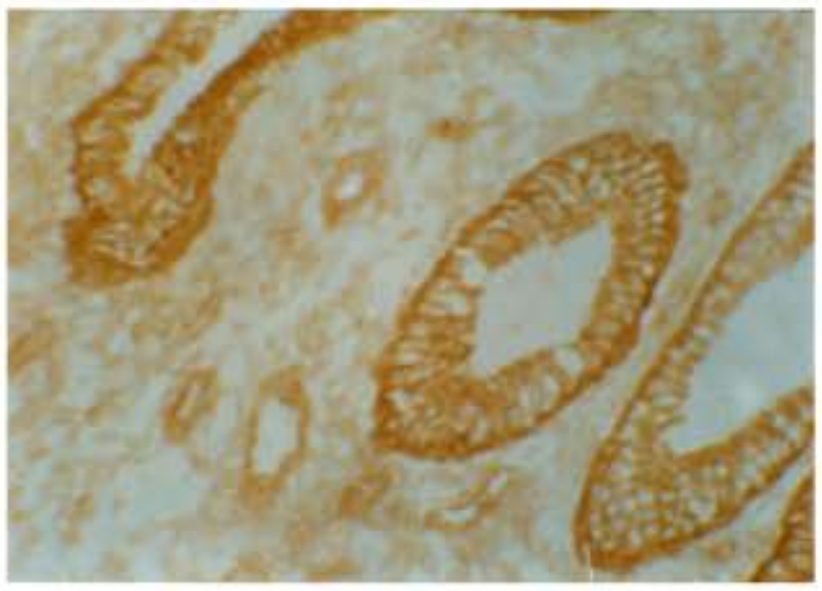

Fig. 4: Frozen section of uterine endometrium tissue of infertile women in secretory phase stained immunohistochemically with anti-VLA-6 monoclonal antibody. The majority of glandular epithelial cells are positive. Stromal cell reactivity is detected. Reactivity with the basement membranes of glandular epithelial cells and vessels is obvious. Magnification x200

VLA-5 insignificantly in both groups. In all cases a minority of lymphocytes and all macrophages for VLA-5 were positive and negative, respectively. More than 50% of glandular epithelial cells in the majority of both groups were positive for VLA-6. Reactivity with vessels for VLA-6 was predominant in all cases of fertile and infertile endometrial tissues (Fig. 4). Less than 25% of stromal cells expressed VLA-6 in both groups. No significant differences were seen between two groups for reactivity of VLA-6 with glandular epithelial cells, vessels, stromal cells ($p = 0.053$, 0.24 and 0.71, respectively). Lymphocytes and macrophages reactivity for VLA-6 were negative.

DISCUSSION

Although the present study showed all the β1 integrin subunits are present in endometrium of fertile and infertile women, there were similarities and differences in the expression of the VLA molecules in different endometrial compartments. In opposite with fertile endometrium tissues, in most unexplained specimens from Iranian women there were no or low reactivity for VLA-4 integrin in glandular epithelial, which suggests this molecule may participate in the cascade of molecular events leading to successful implantation. Differences in VLA-4 integrin expression in nonpregnancy endometrium between fertile and infertile women have been shown by Tabibzadeh (1992), Klentzeris *et al.* (1993) and Skrzypczak *et al.* (2001). No reactivity with anti-VLA-4 antibody in glandular epithelium and stromal cells in endometrium of early pregnancy was found which suggests hormonal dependence in the expression of this molecule throughout the reproductive cycle. The pattern of stromal and epithelial cell reactivity for VLA-4 did not change significantly between early and term pregnancy (Lessey *et al.*, 1994; Lessey, 2000).

The integrins we investigated in this study certainly do not represent the complete family of adhesion molecules, which play a role during implantation. Low expression of other β1 integrin subunits in endometrium of unexplained infertile women has been reported Lessey (2000) and Ghafourian Boroujednia *et al.* (2006). Differences in the expression of α3β1, α4β1 and αvβ1 integrins in endometrium of women with recurrent miscarriages was also detected (Skrzypczak *et al.*, 2001). The endometrial tissues were collected during hysteroscopy performed between 7th and 9th day after ovulation. These authors noted a lower concentration of the α4β1 and αvβ1 in women with recurrent miscarriages integrins during the midluteal phase than in women with unexplained infertility. In other study in endometrial biopsies from women suffering endometriosis β1 subunit was less frequently in the stoma of the endometriosis group than in women without endometriosis. However, α3β1 integrin was more common in the glandular epithelium of women with endometriosis than the other compartments (Szymanowsk *et al.*, 2003). Low expression of α4 was also detected on endometrial glandular epithelial cells in infertile women during implantation time by flow cytometry (Gonzalez *et al.*, 1999).

Differences in integrin expression between in- and out-of-phase endometrial biopsies were observed for αvβ3 integrin in glandular epithelial cells expression during the midluteal phase in women with impaired infertility (Creus *et al.*, 2002). Other studies also showed difference in αvβ3 integrin expression in endometrial stromal cell in subgroups of women with unexplained infertility (Ceydeli *et al.*, 2006; Boroujednia and Nikbakht, 2008). Both αvβ3 and α4β1 integrins have a spatial and temporal expression throughout the implantation window in the endometrium of fertile women (Nardo *et al.*, 2002).

In opposite with above studies, other report showed that there is no significant differences in integrin expression including α4β1, αvβ3 and α1β1 in endometrial biopsies between the IVF group comprising patients with tubal disease, endometriosis and unexplained infertility and the control groups during implantation window. These findings may be related to receiving treatment before IVF, perhaps improving the severity of the disease and therefore, in theory the integrin expression (Thomas *et al.*, 2003).

No significant reactivity for VLA-5 and VLA-6 detected in both fertile and infertile endometrial compartments. The findings for VLA-5 in fertile women were in agreement with other studies (Tabibzadeh, 1992; Klentzeris *et al*., 1993; Bridges *et al*., 1994), expect that Klentzeris *et al*. (1993) did not detect any VLA-5 reactivity and Bridges *et al*. (1994) did not detect vessel and stromal cell reactivity for VLA-5; these discrepancies may be due to use of different antibodies and techniques. It has been shown VLA-6 reactivity with stoma cells increased in normal early pregnancy (Lessey *et al*., 1992, 1994; Ruch *et al*., 1994). VLA-6, which was originally identified on platelets is a laminin receptor (Hemler, 1990). Abundant laminan was detected in endometrium and early pregnancy decidua (Aplin *et al*., 1988). Therefore, it suggests VLA-6 molecules may have a role in pregnancy via interaction with extracellular matrix which leads to activation and release of cytokines.

Integrins play an important role in the endometrial phenotype change that occurs during the secretory phase. At the beginning of pregnancy, the change in integrin expression is synchronized with the trophoblast attachment. Human endometrial epithelium and endothelium are key elements for the initiation of molecular interactions to capture the blastocyst or leukocyte, respectively (Dominguez *et al*., 2005). Some integrins such as αvβ3, α4β1, α6β1 and α7β1 are involved in embryo-endometrium interactions and others (α6β4, α5β1, α1β1, α4β1) in the embryo's invasion of the decidua (Merviel *et al*., 2001). The role of integrins in intrauterine contraception in related to uterine receptivity and embryo implantation are also reported. The change in endometrial integrin expression including VLA-4 and αvβ3 were seen in women using contraception pills and copper intrauterine device (IUD) (Somkuti *et al*., 1996; Savaris *et al*., 2000). Copper IUD can inhibit binding of integrins to the extracellular matrix and it may cause inhibition of the implantation stage, which is crucial for pregnancy (Oruc *et al*., 2005).

Two ligands are known for VLA-4: VCAM-1 and fibronectin. The distribution of VAM-1 in nonpregnant and pregnant endometrium indicates that this adhesion molecule was poorly expressed, particularly in pregnancy (Elices *et al*., 1990). Both VLA-4 and VLA-5 are fibronectin receptors which recognize the CS-1 and RGD alternatively spliced region of fibronectin, respectively (Guan and Hynes, 1990). Abundant fibronectin was detected in endometrium and decidua in vessels, stromal cell cytoplasm and extracellular matrix (Aplin *et al*., 1988). In the present study about 5-50% of lymphocytes for VLA-5 and VLA-4 and macrophages for VLA-4 showed reactivity in both fertile and infertile endometrial tissues. Leukocyte numbers in human endometrium change rapidly after ovulation, particularly as a result of gains in CD56 (bright) uterine NK (uNK) cells. Recently it has been shown that expression of α4 integrin increase in CD56 bright cells at ovulation time in peripheral blood of fertile but not infertile women. The induction of α4 integrin in CD56 (bright) cells of fertile women has been reported. Probability, VLA-4 molecule involved in homing of uterine NK from peripheral blood to endometrium (Peralta *et al*., 2008).

A spatial relationship between VLA-4 and VLA-5-positive lymphocytes and macrophages and fibronectin, possibly causes activation and leads to the release of mediators such as cytokines and prostaglandins which are necessary for reproductive cycle. Increased levels of PGE2 in human endometrium in the secretory phase of the menstrual cycle are seen at the time that the message level of IL-1 increased (Lindhard *et al*., 2002). PGE2 clearly enhances both β1 and β3 integrin subunit expression (Pierro *et al*., 2003). TGFβ, GM-CSF, IFNγ, TNFα and IL-1β have all been demonstrated to alter the expression of integrin molecules (Tabibzadeh, 1991). mRNA and proteins of numerous cytokines and their receptors are expressed in the endometrium (Tabibzadeh, 1994). IL-11 and leukemia inhibitory factor (LIF) are essential for murine implantation and signaling via intracellular phosphorylation (p) of STAT3 in the endometrium. Administration of polyethylene glycol as a potent LIF antagonist on mouse resulted complete implantation failure (White *et al*., 2007). Endometrial pSTAT3 and IL-11 reduced in some women with unexplained infertility (Salamonsen *et al*., 2007). Hence, cytokines may involved in the induction of changes in the endometrial expression of integrins during the reproductive cycle and consequently these changes affect implantation process as confirmed by other studies (Achache and Revel, 2006; Halbersztadt *et al*., 2006).

CONCLUSION

Lack of expression of VLA-4 in the endometrium of infertile woman may result in incomplete embryonal recognition which leads to implantation failure. Further studies focusing on integrin expression within uterine endometrium in conditions that the synchrony of integrin expression is lost will aid our understanding of female infertility.

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