

Endometrial expression of $\beta 3$ integrin, calcitonin and plexin-B1 in the window of implantation in women with unexplained infertility

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Abstract

Background: Endometrial receptivity plays a key role in the establishment of successful implantation and its impairment may contribute to subfertility and limit the assisted reproduction techniques (ART) success.

Objective: The aim of present study was to investigate endometrial receptivity in terms of $\beta 3$ integrin, calcitonin and plexin-B1 expression in women with unexplained infertility.

Materials and Methods: We evaluated expression of $\beta 3$ integrin, calcitonin and plexin-B1 through mRNA level measurement with real-time RT-PCR, in the endometrium of 16 infertile women with unexplained infertility and 10 fertile women. Endometrial biopsies were collected during a single menstrual cycle on postovulatory day LH+7 in each subject.

Results: Significant differences regarding $\beta 3$ integrin and calcitonin expression levels found between patients with unexplained infertility and the fertile women. Endometrial plexin-B1 expression levels showed no significant difference between fertile and infertile women. There were significant correlations between expression of $\beta 3$ integrin with calcitonin and plexin-B1 in fertile and infertile women.

Conclusion: Reduced in endometrial expression of $\beta 3$ integrin and calcitonin alone or together may contribute to unexplained infertility and these genes could account as the potential molecular markers of infertility.

Key words: Unexplained Infertility, Implantation, $\beta 3$ integrin, Calcitonin, Plexin-B1.

This article extracted from M.Sc. thesis. (Maryam Mirani)

Introduction

A critical step in the establishment of pregnancy in natural and assisted human reproduction is embryo implantation (1). Implantation involves complex and sophisticated molecular and cellular interactions between the blastocyst and the receptive endometrium (2). Endometrium is receptive to the embryo for a short period i.e. six days after ovulation and remains receptive for four days known as the window of implantation (3). During the menstrual cycle, several biomarkers such as hormones, receptors, cell-cell adhesion molecules, extracellular matrix proteins, growth factors, cytokines and angiogenic factors mediate morphological and physiological alterations of endometrium which allows the reception of a blastocyst and the establishment of implantation (4). Endometrial receptivity plays a key role in the

establishment of successful implantation and its impairment may contribute to subfertility and limit assisted reproduction techniques (ART) success (5).

It has been suggested that unexplained infertility is due to disturbances in molecular and cellular biomarkers involved in endometrial receptivity (6). In this regard, $\beta 3$ integrin, calcitonin and plexin-B1 have been proposed as potential markers of endometrial receptivity. Endometrial $\beta 3$ integrin has been identified as a cell adhesion receptor whose expression has been shown to be elevated at the time of implantation (7) and proposed as a useful marker of implantation (8). Blockade of the endometrial $\beta 3$ integrin using intrauterine injection of various bioactive compounds caused implantation failure in mice (9). An association has been shown between the aberrant expression of $\beta 3$ integrin and certain types of female infertility (10, 11). Calcitonin, is transiently produced in the uterine epithelia

during the period of implantation (12). Attenuation of calcitonin expression during the preimplantation phase significantly decreases embryo implantation rates in rats and the administration of exogenous calcitonin could promote implantation after embryo transfer (12, 13).

It was found that calcitonin promotes the outgrowth of trophoblasts on human endometrial epithelial cell (EEC), and also, modulates the expression of certain genes in the endometrium, including down-regulating the E-cadherin expression in rodent uterine epithelium and up-regulating of $\beta 3$ integrin in human endometrial carcinoma cell line (13-15). Plexin-B1 is a transmembrane semaphorines receptor implicated in the control of cell migration, angiogenesis and epithelial morphogenesis (16). Plexin-B1 is suggested to exhibit a cyclic pattern in endometrium and to have a role in endometrial receptivity (17, 18).

Evaluation of implantation markers may help to predict pregnancy outcome and detect occult implantation deficiency (19). There are still no reports for the expression of calcitonin and plexin-B1 in the pre-implantation endometrium of women with unexplained infertility. It is reported that endometrial expression of $\beta 3$ integrin was lower in patients with unexplained infertility than in fertile women (8). The role of $\beta 3$ integrin in implantation process is still controversial (20-24). The identification of biomarkers of endometrial receptivity has provided not only information about the molecular mechanisms underlying implantation and a means to investigate the causes of implantation failure but also a potential to be utilized these effectors for developing novel means to improve the receptivity of endometrium (5).

Thus, present study was undertaken to investigate whether $\beta 3$ integrin, calcitonin and plexin-B1 expression alter at the window of implantation in endometrium of infertile patients with unexplained infertility.

Materials and methods

This case-control study was performed in the Laboratory of Embryology, Department of Biology, Shahid Chamran University, Ahvaz, Iran from April 2012 to October 2013.

Sample collection

Endometrial samples were collected using a Novak curette in mid-luteal phase at day

LH+7, from healthy volunteers women with proven fertility (n=10, age 32.5 ± 3.2 yr) and women with unexplained infertility (n=16, age 31.6 ± 3.0 yr) attending the hospital for treatment of infertility. Sample size was determined based on previous studies and because of the ethical considerations and difficulty in sampling was smaller in fertile group (25, 26). Concentration of luteinizing hormone (LH) in morning urine (ACON Laboratories, Inc., San Diego, USA) used to determine the day of the surge.

All women had normal ovarian function and regular menstrual cycles with confirmation of menstrual history, and none of them had used steroid hormones for at least six months prior to the study and used intra-uterine contraceptives. Moreover, all women showed normal tubal patency and no recognizable endometriosis according to symptoms and clinical examination in transvaginal ultrasonography or diagnostic laparoscopy were seen. Women with unexplained infertility had partners with normal values of semen analysis according to the WHO criteria (27). Endometrial samples were divided into two parts. One sample was fixed in 10% formalin and embedded in paraffin. After tissue processing, 5-6 μ m sections were stained with haematoxylin-eosin, evaluated histologically to correspond all samples to the assumed time in the cycle according to the Noyes *et al* criteria (28).

Serum hormone levels

Serum levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E_2) and progesterone (P_4) were measured by the use of commercially kits (Abcam plc, Cambridge, UK).

RNA extraction

Total RNA were extracted from endometrial tissues (approximately 50-100mg) using Tripure (Roche Diagnostics, Germany) according to the recommended protocol by the manufacturer. RNA integrity was analyzed by electrophoresis and total RNA concentrations were obtained using a spectrophotometer at an optical density of 260 nm. The RNA was stored at -70°C for future procedures.

cDNA synthesis

Synthesis of cDNA was carried out from 1mg of total RNA from each sample with

random hexamer primers using prime Script™ RT reagent Kit (Takara Bio Inc., Japan) according to the manufacturer's instructions.

Quantitative real-time reverse transcription polymerase chain reaction analysis (RT-PCR)

Real-time RT-PCR was performed for relative quantification of the $\beta 3$ integrin, calcitonin and plexin-B1 genes expression using ABI StepOne plus™ System (Applied Biosystems, Germany). Hypoxanthine phosphoribosyltransferase (HPRT) gene was used as housekeeping gene. Forward and reverse primer sequences for each gene are presented in Table I. The specificity of primers for each gene was analyzed in BLAST database (29). The reaction mixture consisted of 10 μ l Master mix SYBR Green, 2 μ l cDNA, 1 μ l of each primer (10pmol/ μ l) and 7 μ l dH₂O (Qiagen, Hilden, Germany).

The standard cycling protocol used for all genes consisted of DNA denaturation and enzyme activation at 95°C for 10 min, denaturation 95°C for 15sec, annealing at 62°C for 15 sec and extension and fluorescence acquiring at 72°C for 15 sec. A total of 40 cycles was done. Melting curve analysis was performed by bringing temperature from 95°C to 60°C for 60 sec at the transition rate of 1 degree per second. As Livak and Schmittgen (2001) described, for sample analysis the threshold was set based on the exponential phase of products and the $2^{-\Delta\Delta CT}$ method was performed to analyze the data (30).

Ethical Consideration

This study was approved by the Research Ethics Committee of Shahid Chamran University of Ahvaz, Iran. Written informed consent was obtained from each participant.

Statistical analysis

Data were analyzed by SPSS 16 software (SPSS Inc., USA). Independent samples student's t-test was performed to compare characteristics and hormonal profile of the fertile and infertile women. Results are expressed as mean \pm SD. Comparison of $\beta 3$ integrin, calcitonin and plexin-B1 expression in the groups was done using Mann-Whitney U-test. Spearman correlation analysis was carried out to investigate the relationship

between variables. The level of significance was set at $p < 0.05$.

Results

The mean age, body mass index (BMI), cycle length, duration of menses and hormonal profile in women of both groups are presented in Table II. There were no significant differences in age of women, body mass index (BMI), cycle length, duration of menses and serum LH, FSH, estradiol and progesterone concentrations between two groups. Microscopic analysis of endometrial biopsies showed that all samples corresponded histologically to the mid-luteal phase of endometrial cycle (Figure 1).

Descriptive statistics of $\beta 3$ integrin, calcitonin and plexin-B1 expressions in mid-luteal endometrium of healthy fertile women and patients with unexplained infertility are shown in Table III. Figure 2 shows the relative expressions of $\beta 3$ integrin, calcitonin and plexin-B1 in mid-luteal endometrium of healthy fertile women and patients with unexplained infertility. Relative quantification of $\beta 3$ integrin, calcitonin and plexin-B1 mRNA is based on the expression levels of the reference gene, HPRT.

Levels of $\beta 3$ integrin mRNA expression in endometrium of patients with unexplained infertility were significantly lower than those in fertile women ($p = 0.043$) (Figure 2). Also, calcitonin mRNA levels were significantly higher in the healthy fertile control group compared to infertile women ($p = 0.035$) (Figure 2). While, there was no significant difference in endometrial plexin-B1 mRNA expression in patients with unexplained infertility compared to fertile women ($p = 0.863$) (Figure 2).

Statistically significant correlation was found between $\beta 3$ integrin and calcitonin mRNA expression levels in fertile women ($r = 0.467$, $p = 0.038$) and in patients with unexplained infertility ($r = 0.635$; $p < 0.01$) (Figure 3). Also, there was a significant correlation between $\beta 3$ integrin and plexin-B1 mRNA expression levels in the healthy fertile control group ($r = 0.647$; $p = 0.002$) and in infertile women ($r = 0.706$; $p < 0.01$) (Figure 3). A significant correlation was observed between calcitonin and plexin-B1 mRNA expression levels only in patients with unexplained infertility ($r = 0.580$; $p = 0.001$) (Figure 3).

Table I. Primer sequences used in real-time RT-PCR

Gene	Forward primer(5'→3')	Reverse primer(5'→3')	Accession number
β3 integrin	CATGAAGGATGATCTGTGGAGC	AATCCGCAGGTTACTGGTGAG	NM-000212
Calcitonin	TCTAAGCGGTGCGGTAATCTG	TGTTGAAGTCTGCGTGTATG	NM-001033952
Plexin-B1	ACCAACTGCATTCACCTCCAA	GCATCATCAGGCATCACAG	XM-011533837
HPRT	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	NM-000194

HPRT: Hypoxanthine phosphoribosyltransferase.

Table II. Characteristics and hormonal profile of the fertile and infertile women in the mid-luteal phase

Parameter	Fertile women (n=10)	Infertile women (n=16)	p-value
Age (yrs)	31.7 ±5.9	32.2±5.5	NS [†]
BMI (Kg/m ²)	23.7±2.8	23.4±2.6	NS
Cycle length (days)	28.2±1.3	28.5±1.5	NS
Menses duration (days)	4.2±0.5	4.5±0.6	NS
LH (mIU/mL)	12.54±6.85	13.27±7.13	NS
FSH (mIU/mL)	5.90±2.62	6.58±2.50	NS
Estradiol (pg/ml)	139.3±55.4	142.9±61.6	NS
Progesterone (ng/mL)	10.93±3.21	11.48±4.86	NS

Independent samples t-test was done as the test of significant. Results expressed as mean±SD. The level of significance was set at $p<0.05$. [†]: Non significant.

BMI: Body mass index

LH: Luteinizing hormone

FSH: Follicle stimulating hormone.

Table III. Descriptive statistics of β3 integrin, calcitonin and plexin-B1 expressions in mid-luteal endometrium of fertile and infertile women

Statistics	β3 integrin		Calcitonin		Plexin-B1	
	Fertile	Infertile	Fertile	Infertile	Fertile	Infertile
Mean	79.31	34.42	40.53	16.25	15.93	11.72
Median	23.63	8.89	1.44	0.23	2.92	3.74
SD	1.528E ₂	6.863E ₁	1.677E ₂	7.454E ₁	4.506E ₁	2.524E ₁
Q25	14.32	2.64	0.17	0.02	1.56	1.62
Q50	23.63	8.89	1.44	0.23	2.92	3.74
Q75	61.88	39.85	2.80	0.955	11.59	10.75

SD: Standard deviation

Q: Quartile.

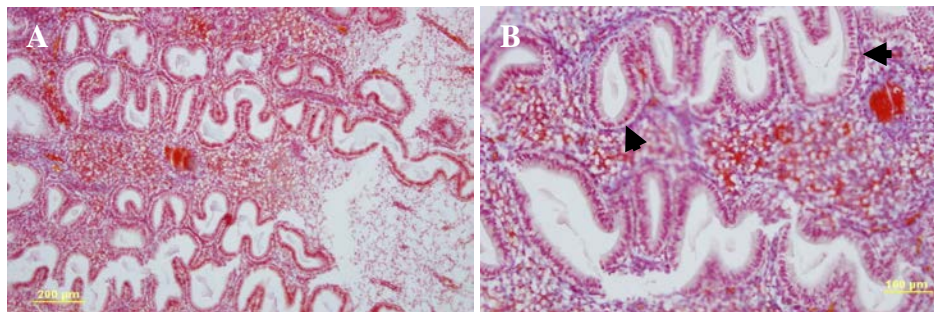


Figure 1. Histological sections of endometrium at mid-luteal phase (A: Scale bar=200 µm, B: Scale bar=100 µm, H&E). Stromal edema and coiled endometrial glands contain secretions with subnuclear vacuolization (black arrows) in their epithelium exhibits endometrium in mid-luteal phase.

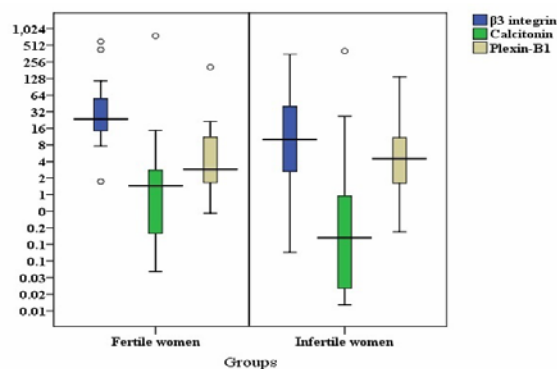


Figure 2. Relative expressions and co-expressions of β3 integrin, calcitonin and plexin-B1 in mid-luteal endometrium of patients with unexplained infertility (n=16) and healthy fertile women (n=10) as revealed by real-time RT-PCR.

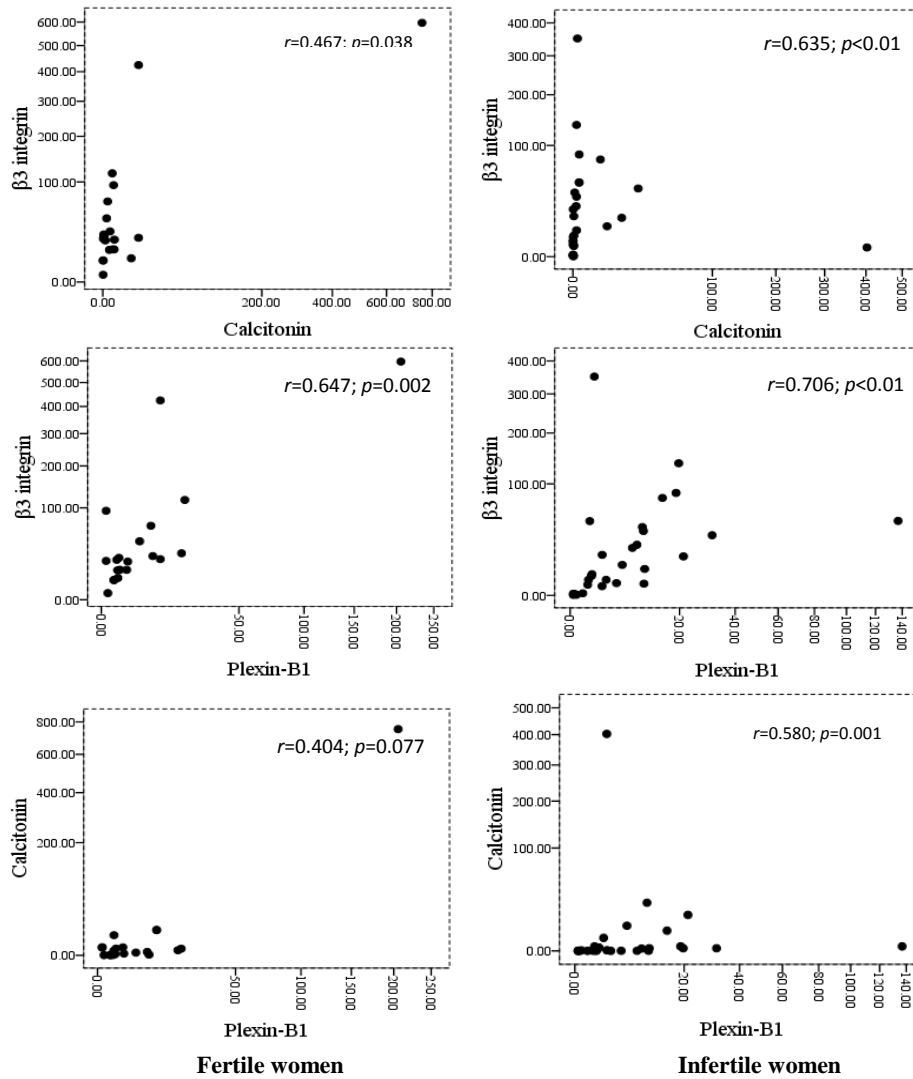


Figure 3. Spearman correlations between $\beta 3$ integrin, calcitonin and plexin-B1 expressions in mid-luteal endometrium of healthy fertile women and patients with unexplained infertility.

Discussion

A receptive endometrium plays a key role in the successful embryo implantation (31). Impaired endometrial receptivity is believed to be a considerable cause of the failure in establishment of pregnancy (32). Understanding of several endometrial growth factors, cytokines and adhesion molecules involved in the implantation might be useful for improving the endometrial receptivity to increase pregnancy rates (1). Current study undertaken to compare the expression levels of some suggested markers of endometrial receptivity in women with unexplained infertility and fertile control.

Present study indicated that $\beta 3$ integrin expression in women with unexplained infertility is impaired in the window of

implantation. Healthy fertile women showed 2.3-fold higher $\beta 3$ integrin mRNA expression than patients with unexplained infertility. $\beta 3$ integrin proposed as a useful marker of the endometrial receptivity. During the menstrual cycle, maximal expression of $\beta 3$ integrin in fertile women has been found in the implantation window (10). Blockade of the endometrial $\beta 3$ integrin causes implantation failure (9).

However, there are conflicting data about $\beta 3$ integrin expression in endometrium of women with unexplained infertility. Some authors reported that there is no difference in $\beta 3$ integrin expression in women with recurrent pregnancy loss (RPL) compared to fertile women (33-35). Nevertheless, Lessey *et al*, Tei *et al*, Othman *et al* and Germeyer *et al* found that women with unexplained RPL

have reduced $\beta 3$ integrin expression compared to controls (8, 10, 36, 37). DuQuesnay *et al* found that $\alpha v\beta 3$ integrin mRNA expression reduced in mid-luteal phase in women with unexplained infertility (26).

Immunohistochemical studies showed that $\beta 3$ integrin is reduced in patients with unexplained infertility in comparison with healthy controls (38-40). In addition, flow cytometric analysis showed that unexplained infertile women expressed lower concentrations of $\beta 3$ integrin in mid-secretory phase (22). However, there are no statistically significant differences regarding $\alpha v\beta 3$ integrin expression between infertile patients with unexplained infertility (24, 26) and endometriosis (41) compared to control women. The discrepancies that observe in expression patterns of $\beta 3$ integrin could be related to technical differences.

Present study also showed down-regulation of endometrial calcitonin in the mid-luteal phase in women with unexplained infertility compared to the fertile controls. Calcitonin mRNA expression in healthy fertile group was 2.4-fold higher than infertile women. Calcitonin is expressed in the human uterine epithelium during the implantation window and has been suggested that it is one of the uterine receptivity biomarkers. It has been indicated also that the expression of calcitonin in the uterus is regulated by progesterone (12). Calcitonin may facilitate uterine receptivity by down-regulating the E-cadherin expression in rodent uterine epithelium and by inducing the tTGase expression in human endometrial epithelial cells (EECs) (15).

Moreover, calcitonin increases the expression of $\beta 3$ integrin directly and also indirectly by stimulation the heparin binding-epidermal growth factor (HB-EGF) and leukemia inhibitory factor (LIF) in human EECs (13, 41). Calcitonin regulates the functions of EECs through calcium mobilization and protein kinase C (PKC) activation (14, 15, 42). Zhu *et al* reported that intrauterine administration of antisense oligodeoxynucleotides against calcitonin mRNA during the preimplantation phase significantly decreases embryo implantation rates in rats (43). In this regard, it has been shown that the injection of calcitonin in the preimplantation phase increases the total

number of implantation sites (13). Moreover, in present study levels of plexin-B1 expression in the window of implantation was lower in infertile women but the difference was not statistically significant. Harduf *et al* showed the involvement of plexin-B1 in trophoblast attachment (17).

The correlation between plexin-B1 expression and estrogen responsiveness in breast cancer cells shows the possible involvement of steroid hormones in plexin-B1 regulation (44). Significant higher expression levels of plexin-B1 at the period corresponding to the implantation window compared to days 12-14, suggest its possible steroid regulation and role in endometrial receptivity (18). Evron *et al* reported that progesterone treatment significantly increases plexin-B1 mRNA and protein levels in endometrial cell cultures and suggested a possible role for plexin-B1 in the trophoblast-epithelial endometrial adhesion process (44). Furthermore, in present study we found positive correlations between $\beta 3$ integrin, calcitonin and plexin-B1 expression at the window of implantation in both fertile and infertile women. Implantation process is a complex and multifactorial event, with association and interplay of the different factors involved. It is important to know how different markers of implantation correlate with each other (1).

It can be suggested that the positive correlations observed between $\beta 3$ integrin, calcitonin and plexin-B1 expression levels could be due to both intricate interactions between these genes or similarity of factors that regulate the expression of them which remain to be explored.

Conclusion

In conclusion, according to our results, $\beta 3$ integrin and calcitonin expression in the window of implantation reduce in women with unexplained infertility. The low expression of $\beta 3$ integrin and calcitonin in women with unexplained infertility supports the possible role of these molecules in the endometrial receptivity during implantation. Therefore, these genes could account as the potential molecular markers of infertility. Plexin-B1 expression in the window of implantation does not impair in women with unexplained infertility. However, further studies are needed to determine the role of $\beta 3$ integrin, calcitonin

and plexin-B1 in receptivity of endometrium during the window of implantation. The identification of endometrial receptivity biomarkers and understanding the mechanisms of their relationships in endometrial receptivity may explain the phenomenon of pregnancy loss and provide new therapeutic strategies for unexplained infertility.

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Conflict of interest

The authors declare that there is no conflict of interest.

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