The Role of Transforming Growth Factor β_2 Gene Expression as a Predictor of Implantation Failure

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Abstract: Objective: To evaluate the possible relationship between transforming growth factor β2 (TGF β2) gene expression within the endometrial tissue and implantation failure in patients with failed intracytoplasmic sperm injection (ICSI). **Patients and Methods:** The current prospective cross sectional study was conducted at Ain Shams University Maternity Hospital during the period between October 2008 and August 2010. Fifty patients with primary unexplained infertility and had previous failed one or more ICSI trials, done in Ain Shams University Maternity Hospital, 3 cycles before the study. Endometrial samples were obtained without anesthesia from each women using Pipelle® biopsy. Endometrial biopsy was taken at day 18-23 from the cycle. TGF-β2 gene expression was evaluated in the endometrium of secretory phases by immunohistochemistry with scoring through two independent observers regarding intensity of immunostaining in each of glandular epithelium, luminal epithelium and stroma as well as spread (surface area) of immune reaction. The immunostaining intensity scores were: Zero for absent staining, one for mild staining, two for moderate staining and 3 for strong staining. After sample all cases underwent one cycle of ovarian hyperstimulation completed by ICSI. The included women were divided into 2 groups according to ICSI results: Group A: Included infertile women who became pregnant and group B: Included infertile women who had another failed ICSI cycle. **Results:** A total of 50 pregnant women with primary infertility and previous failed ICSI were included in the study. The included women were divided in to 2 groups according to ICSI results: Group A: Included 12 infertile women who become pregnant and group B: Included 38 infertile women who had another failed ICSI cycle. There was a non-significant difference (p>0.05) between both groups regarding the mean age of patients, body mass index (BMI),and duration of infertility, basal follicular stimulating hormone (FSH) and luteinizing hormone (LH), thyroid stimulating hormone(TSH), prolactin and progesterone but significant difference (p<0.01) in endometrial thickness . As regards endometrial thickness; it showed high significant correlation (p<0.01) with TGFβ2 immunostaining intensity in luminal epithelium, glandular epithelium, stromal cells and total surface area staining. There was a significant difference (p<0.01) between both groups as regards TGF2β immunostaining intensity in luminal epithelium, stromal epithelium glandular epithelium, and also in total surface area. Regarding immunostaining intensity scores the specificity failure percent in luminal epithelium, glandular epithelium, stromal cells and total surface area, if the score ≥ 2.50, was 90%, while sensitivity failure in luminal epithelium, glandular epithelium, and total surface area, was 87.7%.If immunostaining intensity score was ≥ 0.50 the specificity failure was 0% in glandular epithelium and total surface area while the sensitivity failure was 50%. **Conclusion:** Endometrial TGFβ2 expression can be used as an investigation for couples with repeated failed ICSI. It can be also used as a marker for optimal implantation, especially before ICSI trials.

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1. Introduction:

The study of uterine receptivity in women relies primarily on non-invasive methods, such as ultrasonography. However, the predictive value and the reproducibility of these methods remain limited, and new methods of assessing uterine receptivity are needed. Uterine flushing and endometrial biopsy have been used to assess various types of factors involved in the implantation process [1]. The cyclical endometrial activity is regulated by the ovarian steroids estrogen and progesterone, but at a paracrine level by a myriad of growth factors, cytokines and proteases. Unsurprisingly, transforming growth factor (TGFβ) superfamily members are abundantly and dynamically expressed in the endometrium, and appear, through their actions associated with cell proliferation, differentiation, apoptosis and tissue remodeling, to have instrumental roles in modulating cellular events involved in menstruation, proliferation, decidualization and the establishment of pregnancy [2]**.** The aim of the present study was to evaluate the possible relationship between transforming growth factor β2 (TGF β2) gene expression within the endometrial tissue and implantation failure in patients with failed intracytoplasmic sperm injection (ICSI).

2. Patients and Methods

The current prospective cross sectional study was conducted at Ain Shams University Maternity Hospital during the period between October 2008 and August 2010. Fifty patients aged 20-35 years with primary unexplained infertility were included in the study. Primary infertility was defined as inability to conceive with continuous marital life for at least one year. Unexplained infertility was diagnosed by exclusion of known factors of infertility. Ovulation was confirmed with follicular monitoring by transvaginal ultrasound and/or mid-luteal progesterone ≥5ng/ml. Tubal patency was confirmed by hysterosalpingography and or pelvic laparoscopy and male factor infertility was excluded by semen parameters meeting the World Health Organization (1999) criteria [3, 4]. Included women had previous failed one or more ICSI trials, done in Ain Shams University Maternity Hospital, 3 cycles before the study. Exclusion criteria were women with secondary infertility, polycystic ovarian syndrome, follicular stimulating hormone (FSH)>10mIU/ml, endometriosis, hypo- or hyperthyroidism, diabetes mellitus , hyperprolactinemia, age less than 20years or more than 35years,Cushing syndrome and /or if embryos transferred during ICSI were less than three or of grade other than grade A or difficult transfer. Trans-vaginal sonography was done starting from day 2 of the cycle. Transvaginal sonography was performed, using transvaginal transducer (7.5 MHz, Medison Sonoace X_6, Korea). During scanning, the number/size of follicles and the endometrial thickness were recorded.

Endometrial samples were obtained without anesthesia from each women using Pipelle® biopsy. Endometrial biopsy was taken at day 18-23 from the cycle. All endometrial biopsies were fixed in 10% formaldehyde, embedded in paraffin blocks, and stained with Haematoxylin and Eosin for histopathological assessment and histological dating, together with morphometric analysis, then Immunohistochemical staining for transforming growth factor β2 using monoclonal antibody.

TGF-β2 expression was evaluated in the endometrium of secretory phases by immunohistochemistry. The positive control specimen was examined for a brown-colour end product at the site of the target antigen. The presence of this colour can be interpreted as a positive staining result indicating that the kit reagents are performing properly. In negative control specimen, there was absence of specimen staining.

Interpretation of results by Light microscopy: After the staining process, the specimens were observed under microscopy. Intact cells were only used for interpretation since necrotic or degenerated cells often stain non-specifically. Precipitates may form if, for example, specimens were allowed to dry out during the staining procedure. This was apparent at the edge of the specimen. Using of 40x magnification for scanning minimized this potential mis-interpretation. Cells labelled by the antibody displayed a staining almost entirely confined to the cell membrane. The cell membrane expression of TGFβ2 (immunostaining) was identified in every endometrial section as regards: Immuno-staining intensity in each of: luminal epithelium, glandular epithelium, stromal cells and Immuno-staining spread in the whole field. Two independent observers evaluated each sample regarding intensity of TGFβ2 immunostaining .The immunostaining intensity scores were: Zero for absent staining, 1 for mild staining, 2 for moderate staining and 3 for strong staining. After endometrial samples, all cases underwent one cycle of ovarian hyperstimulation completed by ICSI. The included women were divided into 2 groups according to ICSI results: Group A: Included infertile women who become pregnant and group B: Included infertile women who had another failed ICSI cycle.

Statistical analysis

Statistical analysis was performed using Microsoft® Excel® version 2010 and Statistical Package for Social Sciences (SPSS®) for Windows® version 15.0. Data was described as mean and standard deviation (for numeric continuous variables), number and percentage (for categorical variables). Difference between two groups was estimated using independent student's *t*-test. Pearson correlation analysis assessing the strength of association between two variables. The correlation co-efficient denoted symbolically r, defines the strength and direction of the linear relationship between two variables.A propability (p) value less than 0.05 was cosidered statestically Significant . Diagnostic validity test included: a. The diagnostic sensitivity: It is the percentage of diseased cases truly diagnosed (TP) among total diseased cases. b. The diagnostic specificity: It is the percentage of non-diseased truly excluded by the test (TN) among total non-diseased cases. c. The predictive value for a +ve test: It is the percentage of cases truly diagnosed among total positive cases.d. The predictive value for a -ve test: It is the percentage of cases truly negative among total negative cases.e. The efficacy or the diagnostic accuracy of the test: It is the percentage of cases truly diseased plus truly non-diseased among total cases. The ROC was constructed to obtain the most sensitive and specific cutoff for each technique. To evaluate the most discriminating markers between the compared groups, area uder the curve (AUC) can also be calculated.

3.Results

A total of 50 pregnant women with primary infertility and previous failed ICSI were included in the study. The included women were divided into 2 groups according to ICSI results: Group A: Included 12 infertile women who became pregnant and group B: Included 38 infertile women who had another failed ICSI cycle. There was a non-significant difference (p>0.05) between both groups regarding the mean age of patients, body mass index (BMI),and duration of infertility, basal FSH and LH, TSH, Prolactin and progesterone but significant difference (p<0.01) in endometrial thickness (Tables 1,2). As regards endometrial thickness; it showed high significant correlation (p<0.01) with TGFβ2 immunostaining intensity in luminal epithelium, glandular epithelium, stromal cells and total surface area staining. There was a significant difference (p<0.01) between both groups as regards TGF2β immunostaining intensity in luminal epithelium, stromal epithelium glandular epithelium, and also in total surface area (Table3). Regarding immunostaining intensity scores the specificity failure percent in luminal epithelium, glandular epithelium, stromal cells and total surface area, if the score ≥ 2.50, was 90%, while sensitivity failure in luminal epithelium, glandular epithelium, and total surface area, was 87.7%.If immunostaining intensity score was ≥ 0.50 the specificity failure was 0% in glandular epithelium and total surface area while the sensitivity failure was 50% (Table 4). Correlation between the scoring of TGFβ2 immunostaining parameters versus infertility factors in whole study group is shown in (Table5). Table 6 shows the correlation between intensity of TGFβ2 immunostaining parameters versus the hormonal profile of the study group.

Table (1): Difference between both groups regarding demographic data*

	Group A (n=12)	Group B(n=38)	*P***
Age (Years)	29.06 ± 4.221	30.34±3.479	0.250(NS)
BMI (Kg/m^2)	29.06±2.940	31.34 ± 4.345	0.539(NS)
Duration of infertility (years)	5.06±1.765	6.00±2.463	0.159 (NS)
Endometrial thickness (mm)	13.21±0.577	10.11±1.232	0.006(HS)

*Values are expressed as mean± standard deviation
** Analysis using Independent Student's *t*-Test
BMI body mass index [calculated as the weight (in kilograms) divided by the squared height (in meters)]
NS non-significant HS highly significant

Table (2): Comparison between both groups regarding hormonal profile*

	Group A (n=12)	Group B (n=38)	*P***
FSH (IU/L)	6.25±0.9457	7.05±1.974	0.770(NS)
LH (IU/L)	3.90±0.7963	4.387±1.467	0.421(NS)
TSH (IU/L)	1.306±0.6664	1.637 ±0.816	0.148(NS)
Prolactin (ng/mL)	18.794±4.5779	18.703±6.212	0.957(NS)
Progesterone (ng/ml)	20.639±1.8548	18.912±3.030	0.73(NS)

* Values are expressed as mean± standard deviation ** Analysis using Independent Student's *t*-Test
FSH follicular stimulating hormone, LH luteinizing hormone
TSH thyroid stimulating hormone, NS non-significant

Table (3): Comparison between both groups regarding the scoring of TGFβ2 immunostaining intensity *

	Group A (n=12)	GroupB (n=38)	*P***
Luminal epithelium	1.11±0.471	1.63±0.907	**S**
Glandular epithelium	1.89±0.583	2.13±1.070	**S**
Stromal cells	0.94±0.725	1.47±1.016	**S**
Total surface area	1.67±0.485	2.09±1.088	**S**

* Values are expressed as mean± standard deviation
** Analysis using Independent Student's *t*-Test

Table (4): Table show the sensitivity and specificity of failure in each TGFβ2 immunostaining score*

	Failure if greater than or equal To	Sensitivity	Specificity
Luminal epithelium	≥0.50	**15.6%**	**5.6%**
	≥1.50	59.4%	83.3%
	≥2.50	**87.5%**	**90.8%**
Glandular epithelium	≥0.50	**50.0%**	**0.0%**
	≥1.50	75.0%	22.2%
	≥2.50	**87.5%**	**90.8%**
Stromal cells	≥0.50	**18.8%**	17.8%
	≥1.50	46.9%	77.8%
	≥2.50	**81.3%**	**90.8%**
Total surface area	≥0.50	**50.0%**	**0.0%**
	≥1.50	71.9%	33.3%
	≥2.50	**87.7%**	**90.8%**

*Values are expressed as numbers and percentage (%) TGFβ2 transforming growth factor β_2

Table (5): Correlation between the scoring of TGFβ2 immunostaining parameters versus infertility factors in whole study group.

	Age		Duration of infertility		BMI		Endometrial thickness	
	r	*p**	r	*p* *	r	*p* *	r	*p* *
Luminal epithelium	-0.222	0.121	-0.106	0.465	-0.085	0.555	0.532	<0.01
Glandular epithelium	-0.063	0.664	0.026	0.857	-0.075	0.604	0.503	<0.01
Stromal cells	-0.047	0.743	-0.031	0.832	-0.002	0.988	0.562	<0.01
Total surface area	-0.106	0.463	-0.029	0.841	0.005	0.975	0.579	<0.01

BMI body mass index [calculated as the weight (in kilograms) divided by the squared height (in meters)]
* Analysis using Pearson correlation Test

Table (6): Correlation coefficient between intensity of TGFβ2 immunostaining parameters versus the hormonal profile of the study group*

	FSH		**LH**		**TSH**		**Progesterone**		**Prolactin**	
	r	*p* *	**r**	*p* *	**r**	*p* *	**r**	*p* *	**r**	*p* *
Luminal epithelium	-0.073	0.616	0.035	0.808	0.250	0.081	0.048	0.739	0.249	0.081
Glandular epithelium	0.087	0.550	-0.224	0.118	0.084	0.561	0.075	0.606	**0.372**	**0.008**
Stromal cells	0.172	0.233	0.016	0.911	**0.293**	**0.039**	0.046	0.749	**0.363**	**0.010**
Total surface area	0.074	0.608	-0.170	0.238	0.131	0.365	0.112	0.437	**0.408**	**0.003**

* Analysis using Pearson correlation Test FSH follicular stimulating hormone
LH luteinizing hormone TSH thyroid stimulating hormone

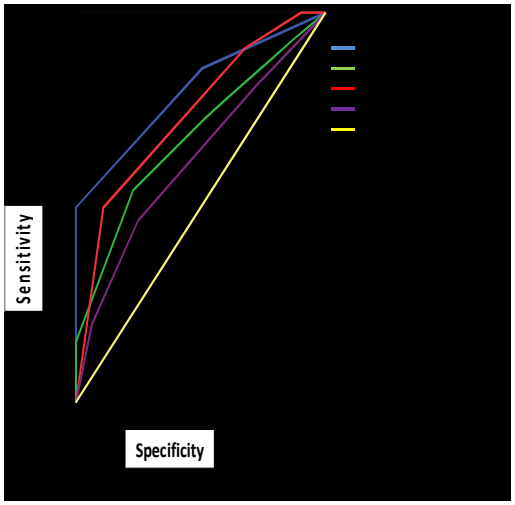

Figure (1): Receiver operator characteristic curve

4. Discussion

In this study, There was no statistically significant difference (p>0.05) between both groups regarding the duration of infertility and BMI also, they were negatively correlated to TGFβ2 immunostaining among all cases (p> 0.05). The above results prove that age, duration of infertility and BMI wasn't affecting TGFβ2 expression in the endometrium at window of implantation. Also, our results proved that both basal serum FSH, LH and progesterone levels were negatively correlated to TGFβ2 expression among the both groups (p >0.05), showing that serum FSH, LH and progesterone may have no role concerning TGFβ2 expression. The above results may signify that TGFβ2 expression is not under control of different hormones as FSH, LH, or progesterone. In our study the level of serum TSH had no significant difference between both groups but, there is significantly positive correlation (p <0.05) between TSH and TGFβ2 immunostaining of

stromal epithelium only. Elena Vaquero et al., reported that the presence of an underlying thyroid abnormality could have an adverse effect upon implantation, compromising the success of in vitro-fertilization (IVF) treatment. They found that since 46% of patients experiencing repeated IVF failures were affected by a mild thyroid abnormality [5]. In current study there wasn't significant difference between both groups in the level of serum prolactin but there was a significant positive correlation (p<0.01) between serum prolactin and TGFβ2 immunostaining intensity of glandular epithelium, stromal cells and the total surface area immunostaining, but there is no significant correlation with luminal epithelium. This indicates the role of prolactin in TGFβ2 expression at time of window of implantation.

Matsuyama *et al., 1990* reported that in rodents, a possible role for TGFβ2 is through mediation of the luteotrophic actions of prolactin and subsequent inhibition of apoptosis of luteal cells. Prolactin enhances progesterone production via the suppression of 20a-hydroxysteroid dehydrogenase (20a-HSD) expression. In cultured rat luteal cells, both prolactin and TGFβ2 suppressed 20a-HSD activity [6].

We found significant positive correlation (p<0.01) between endometrial thickness and TGFβ2 immunostaining intensity of luminal epithelium, stromal cells and the total surface area immunostaining, also it is significant positively correlated (p<0.05) with TGFβ2 immunostaining in glandular epithelium.Other investigators *reported that:* the three TGFβ isoforms are differentially expressed in endometrium; with TGFβ2 predominantly localizing to stroma [7,8].

In the comparison between both groups in the mean of each parameter of TGFβ2 immunostaining intensity we found that there was significant difference (p<0.01) between both groups in luminal epithelium, stromal cells and total surface area, but there is significant difference (p<0.05) with glandular epithelium.

In our study we calculate the sensitivity and specificity of failure in TGFβ2 immunostaining, we found that specificity failure percent in luminal epithelium, glandular epithelium, stromal cells and total surface area, in score ≥ 2.50, it was 90%, while sensitivity failure in luminal epithelium, glandular epithelium, and total surface area, it was 87.7%.

In score ≥ 0.50 we found specificity failure 0% in glandular epithelium and total surface area with sensitivity failure 50%.

From the above statistics we found ascending manner sensitivity and specificity of failure in TGFβ2 immunostaining score. This means the high expression of TGFβ2 the high sensitivity and specificity for failure.

Jana Skrzypczak et al., reported that RT-PCR investigation showed trend for higher expression of TGFβ2 in endometrial samples from women with idiopathic infertility in secretory phase. In 59% of their endometrium they observed higher TGFβ2 expression than in control group. Also in 64% of endometrium from women with unexplained recurrent miscarriages they noticed a higher TGFβ2 expression than in endometrium from women in control group [9]. Also Jana Skrzypczak *et al.,* added that deregulation ofTGFβ2 expression is associated with infertility and early pregnancy loss. However the exact mechanism of how overexpression of endometrial TGFβ2interferes with implantation may be more complex[9]. Caryn et al.,support the role for TGFβ2 as an autocrine/ paracrine regulator for controlling the invasion of decidua by the implanting embryo[10]. The present study suggests that TGFβ2 is related to human fertility and plays an important role in human implantation, as the maximal TGFβ2 expression is in the luteal phase (day18-23), which co-insides with the time of human implantation. Also those disturbances in endometrial extracellular matrix and deregulated expression of TGFβs genes during the implantation window might be a cause of impaired implantation in repeated ICSI cycles.

Conclusion

Endometrial TGFβ2 expression can be used as an investigation for couples with repeated failed ICSI. It can be also used as a marker for optimal implantation, especially before ICSI trials.

Declaration of interest

The authors reported no conflict of interest.All of the authors had substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting and revising the article critically with final approval of the version to be published. The research was funded by the authors.

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