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Implantation Failure Is Associated With Increased α-Inhibin and β-Glycan Gene Expression in Secretory Phase Endometrium: Nested Case–Control Study of Infertile Women Undergoing IVF/Fresh Embryo Transfer

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Abstract

Embryo implantation involves a complex sequence of events, and a large amount of molecules have been postulated to be involved in the interaction of embryo and endometrium. This study evaluated the endometrial expression of α-inhibin and β-glycan in the mid-secretory phase of women scheduled to in vitro fertilization (IVF) and tested whether these markers are associated with implantation failure. We performed a nested case–control study including 52 women submitted to IVF and embryo transfer, divided into 2 groups: cases with implantation failure (n = 33) and controls with confirmed clinical pregnancy (n = 19). Endometrial α-inhibin and β-glycan gene expression was evaluated in the mid-secretory phase of the natural menstrual cycle immediately before IVF, using real-time polymerase chain reaction. We found a higher gene expression of α-inhibin (fold increase = 2.14 ± 0.32, $P < .05$) and β-glycan (fold increase = 1.44 ± 0.16, $P < .05$) in implantation failure patients compared to confirmed clinical pregnancy patients. The areas under the receiver operating characteristics curves for prediction of implantation failure in this context were 0.692 and 0.678 for α-inhibin and β-glycan, respectively. The present results suggest that high expression levels of α-inhibin and β-glycan transcripts in secretory phase endometrium are associated with a lower chance of achieving pregnancy with IVF.

Keywords

growth factors, TGF-β superfamily, endometrial receptivity

Introduction

In human reproduction, successful embryo implantation relies on a healthy embryo, a receptive endometrium, and the synchronization of both.[1] Embryo implantation is a challenge to medically assisted reproduction, and its failure is considered the major cause of reproductive wastage in natural and in assisted conception.[2] Embryo implantation involves a complex sequence of events, and a large amount of molecules have been postulated to be involved in the interaction of embryo and endometrium.[3]

Inhibin is a dimeric glycoprotein of the transforming growth factor β (TGF-β) superfamily, a class of peptides that regulate the growth and differentiation of a variety of cell types and tissues, including the endometrium. Inhibin is a potent competitive antagonist of activin, as it binds the extracellular portion of activin type II receptor in a conformation that blocks the receptor signal transmission. Inhibin action is amplified by a membrane-anchored proteoglycan named type III TGF-β receptor or β-glycan, which binds the α subunit of inhibin and acts as a coreceptor.[4-6]

Both inhibin and β-glycan are endometrial factors expressed across the menstrual cycle and pregnancy.[7-9] Inhibin α-subunit is detected in normal human endometrium, localized predominantly in the glandular and luminal epithelium.[10,11] β-Glycan is localized in stromal and epithelial cells and is particularly abundant in the basal and apical borders of the glandular epithelium.[12] β-Glycan has also been found in decidual cells obtained from the first and the third trimesters of pregnancy and in endothelial cells of early pregnancy.[13]

Considering the multiple physiological targets of activins and inhibins in the endometrium and decidua and the

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postulated role of activin in endometrial tissue remodeling, upregulating the expression of matrix metalloproteinases and promoting trophoblast invasion,[14] we hypothesized that abnormal expression of inhibin and/or β-glycan in secretory phase endometrium might be a biomarker of reduced endometrial receptivity to embryo implantation. In order to address this hypothesis, we measured α-inhibin and β-glycan messenger RNA (mRNA) expression levels in secretory phase endometrial samples from women undergoing in vitro fertilization (IVF) and fresh embryo transfer and investigated whether these molecular markers differed between cases of implantation failure and patients with confirmed clinical pregnancy.

Materials and Methods

This matched case–control study (n = 52) was nested in a cohort of 135 consecutive patients referred for conventional IVF or intracytoplasmic sperm injection (ICSI) at the Division of Human Reproduction of the Academic Hospital of Federal University of Minas Gerais, Belo Horizonte, Brazil, between 2008 and 2010.[15] The recruitment occurred when the patient had her first visit in preparation for IVF, and the follow-up occurred until the end of treatment, if pregnancy was not achieved, or the end of pregnancy. Informed consent was obtained from all participants prior to inclusion in the study, which was approved by the local Human Investigation Committee.

To avoid selection bias, the selection of cases and controls was made before the analyses of target genes. Among the 29 confirmed clinical pregnancies, we randomly selected 19 participants to be included in this study as controls. Among the 73 cases of implantation failure, we selected 33 cases matching the control group by woman's age (33 ± 5 years). Obese women (body mass index 30 kg/m^2 or higher) or women older than 40 years were not included.

All patients underwent endometrial biopsy with a disposable cannula of small caliber (Pipelle; Prodimed, Neuilly-en-Thelle, France). Samples were collected between the 18th and the 20th day of the menstrual cycle on the month before treatment and were immediately chilled in liquid nitrogen and stored at −80°C in a 2-mL sterile tube free of RNAse and DNAse for subsequent RNA extraction and analysis of gene expression. The biopsies were performed immediately before the implantation window to avoid the remote risk of intervening on a spontaneous pregnancy. All endometrial biopsies included in this study had adequate material for analysis.

Controlled ovarian hyperstimulation and IVF procedures have been described in detail elsewhere.[15] Briefly, all women underwent a long gonadotropin-releasing hormone agonist protocol with intranasal nafarelin (400 μg daily) for pituitary blockade beginning on day 21 of the previous menstrual cycle. Transvaginal ultrasound was performed to confirm blockade of ovarian function based on endometrial thickness < 4 mm and absence of follicular growth. Controlled ovarian stimulation was performed with recombinant follicle-stimulating hormone (FSH) and/or highly purified human urinary gonadotropin at a dose of 300 IU for 3 days and 150 IU on the following days.

Transvaginal ultrasound was performed every other day beginning on the fifth day of FSH therapy and daily, since the mean follicular diameter reached 14 mm, to monitor the follicular growth. The cycle was cancelled if there were <3 growing follicles. When at least 1 dominant follicle reached a diameter of 18 mm, 250 μg of recombinant human chorionic gonadotropin (hCG) were administered, and the oocyte retrieval occurred approximately 36 hours after hCG injection, using a 17-gauge follicular aspiration needle connected to a transvaginal probe and a Craft suction unit with a negative pressure of 100 mm Hg.

Semen was prepared using the swim-up technique, and insemination was performed by conventional IVF or ICSI. The indications for ICSI were male infertility and male partner infected with human immunodeficiency virus. Oocyte fertilization was assessed at 18 to 20 hours after insemination by confirmation of the presence and location of 2 pronuclei. Morphological scoring of preimplantation embryos, based on blastomere number, symmetry, fragmentation, presence of vacuoles, and characteristics of the zona pellucida, was assessed to classify embryo quality before transfer.[16,17] Up to 4 fresh embryos were selected for transfer, and the remaining were left in the culture medium until the blastocyst stage and subsequently cryopreserved. Micronized progesterone (600 mg/d) was administered vaginally for luteal phase support from the day of embryo transfer until the pregnancy assessment with serum β-hCG 2 weeks after embryo transfer. If a pregnancy occurred, this therapy was extended until 8 weeks of gestation.

A clinical pregnancy was defined as the presence of fetal heart beat by transvaginal ultrasonography around the sixth week of gestation.[15] Implantation failure was defined as a negative pregnancy test 2 weeks after embryo transfer.

RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA was isolated from samples using homogenization in TRIzol reagent (Invitrogen, Carlsbad, California), according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of DNAse I-treated RNA in a reaction volume of 20 μL using SuperScript III reverse transcriptase kit (Invitrogen), according to the manufacturer's instructions. The complementary DNA was subsequently subjected to real-time polymerase chain reaction (RT-PCR) using SYBR Green Master Mix kit (Life Technologies, Invitrogen), with the following reaction conditions: 52°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 15 seconds at 95°C for denaturation and 60 seconds at 60°C for primer annealing. The melting curves showed single amplicons and no primer dimers. Target gene expression was normalized by the reference gene *S26* that encodes a ribosomal protein. Oligonucleotide primers were designed based on the GeneBank NCBI Blast sequence (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) using IDT DNA online tool (http://www.idtdna.com). Primer sequences are shown in Table 1.

Table 1. Primers for Real-Time PCR.

Primers	Nucleotide Sequence (5′-3′)	Primer Size	Amplicon Size	GenBank Accession Number
S26 (sense)	CATTGACCTCACCTTTCACCTGCT	24 nt	96 base pairs	NM_001029.3
S26 (antisense)	TCGAATATGATGCGGTTCTGCTCG	24 nt		
β-Glycan (sense)	GGGAAGATCAAGTGTTCCCTCCAA	24 nt	84 base pairs	NM_003243.3
β-Glycan (antisense)	TGGGTTGAAGGTACTCAGCAAGGT	24 nt		
Inhibin (sense)	ACTGCCACAGAGTAGCACTGAACA	24 nt	87 base pairs	NM_002191.2
Inhibin (antisense)	AGTGGAAGATGAAACTGGGAGGGT	24 nt		

Abbreviation: PCR, polymerase chain reaction; nt, nucleotides.

Statistical Analysis

Clinical data are expressed as median and interquartile interval, and the group medians were compared by the nonparametric Mann-Whitney *U* test. The PCR results were obtained as cycle threshold (C_t) and normalized to ΔC_t, which is the C_t of the target gene minus the C_t of the reference gene, *S26*. The relative gene expression (mean $\pm$ standard deviation) was calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t$ is the ΔC_t of the case group minus the ΔC_t of the control group.[18] Group comparisons were performed using the median ΔC_t values and Mann-Whitney *U* test.

The free software Java Applets for Power and Sample Size (The University of Iowa) and statistical parameters obtained from published studies of similar design[12] were used for power calculation. Sample size calculation indicated that 25 cases and 15 controls would allow the detection of differences of at least 2-fold mRNA expression between the 2 groups with 90% statistical power and 95% confidence interval (CI).

Partial correlation coefficients were calculated to test the linear correlation between the implantation rate and the endometrial expression of α-inhibin and β-glycan, adjusting for potential confounders, namely age, time of infertility, endometrial thickness, and serum anti-Müllerian hormone (AMH) levels. Gene expression was computed as $2^{-\Delta Ct}$, and all preselected variables were entered in the adjusted correlation coefficients. Receiver operating characteristic (ROC) curves were calculated for prediction of implantation failure with their respective 95% CIs. The best cutoffs indicated by ROC curve analysis were used to calculate the positive and negative likelihood ratios.[19] Statistical analysis was carried out using SPSS version 22, and $P < .05$ was considered statistically significant.

Results

The median age was 34 years in the case group and 33 years in the control group ($P = .523$; Table 2). In addition, we confirmed that cases and controls had similar clinical characteristics such as duration of infertility, day 3 serum FSH and AMH levels, antral follicle count, endometrial thickness, dose of gonadotropins used, number of oocytes, fertilization rate, number of embryos obtained, and number of embryos transferred (total and good quality embryos), as shown in Table 2. The proportion of women with endometriosis or unexplained infertility was similar in cases (5 of 33 = 15%) and controls (2 of 19 = 11%), while the remaining participants had male factors such as sperm abnormalities or human immunodeficiency virus infection (67% of cases and 78% of controls) or tubal obstruction (18% of cases and 11% of controls, $\chi^2 = 4.7$, $P = .45$).

Table 2. Characteristics of the Patient Groups.[a]

	Implantation Failure (n = 33)	Clinical Pregnancy (n = 19)	*P*
Age, years	34.0 (31.5-37.0)	33.0 (28.0-39.0)	.523
Duration of infertility, years	7.0 (4.0-10.0)	5.0 (3.0-9.0)	.211
Day 3 FSH, mIU/mL	7.1 (4.8-8.3)	7.4 (6.1-8.2)	.506
Day 3 AMH, ng/mL	0.85 (0.48-2.49)	1.86 (0.74-3.37)	.151
Antral follicle count	8 (6-11)	9 (6-11)	.485
Endometrial thickness, mm	4.0 (4.0-5.0)	4.0 (4.0-5.0)	.433
Dose of gonadotropins used, IU	1800 (1650-1950)	1950 (1800-1950)	.184
No. of oocytes	6 (4-10)	7 (4-11)	.647
Fertilization rate, %	67 (47-82)	55 (50-75)	.164
No. of embryos obtained	3 (2-6)	4 (2-6)	.923
No. of embryos transferred	3 (2-3)	3 (2-3)	.673
Good quality embryos transferred	2 (2-3)	3 (2-3)	.268
Cause of infertility			.450[b]
Unexplained/ endometriosis	5 (15%)	2 (11%)	
Male factors	22 (67%)	15 (78%)	
Tubal obstruction	6 (18%)	2 (11%)	

Abbreviations: AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone.
[a]Data are expressed as medians and interquartile interval. *P* values are for the Mann-Whitney *U* test.
[b]Chi-square test.

All endometrial samples analyzed expressed the target genes. The relative gene expression of α-inhibin in secretory phase endometrial samples from women with subsequent implantation failure was significantly higher than in samples from women who achieved clinical pregnancy (fold increase = 2.14 $\pm$ 0.32, $P < .05$; Figure 1). β-Glycan gene expression was also increased in the endometrial samples from women who ended with implantation failure,

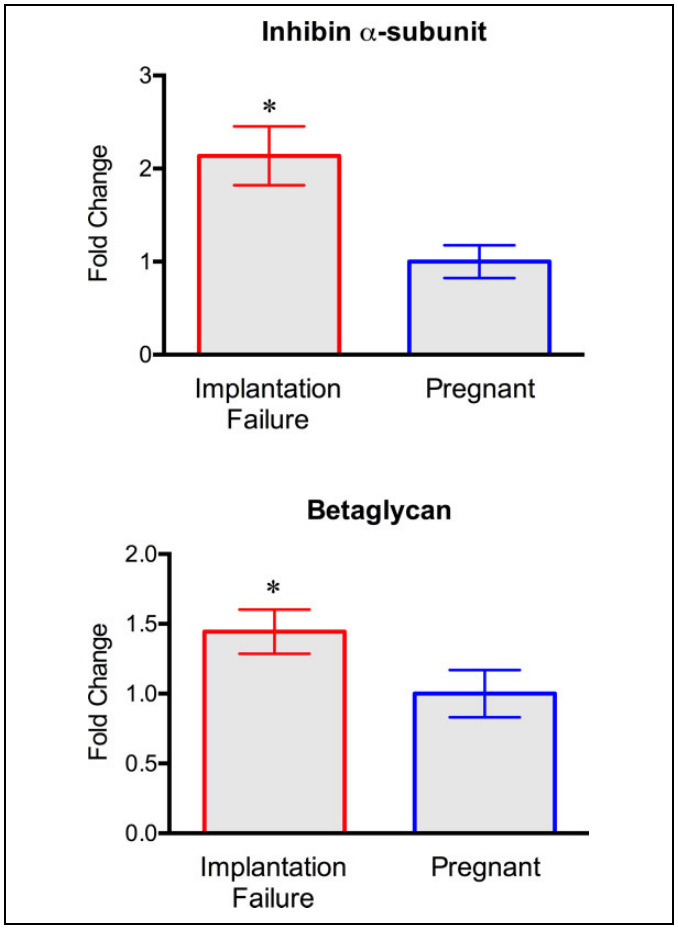

Figure 1. α-Inhibin and β-glycan gene expression levels in endometrial samples obtained in the mid-secretory phase of the spontaneous menstrual cycle immediately before in vitro fertilization with embryo transfer. Data are expressed as fold change (± standard deviation [SD]) of gene expression in the cases (implantation failure, n = 33) relative to controls (clinical pregnancy, n = 19). *$P < .05$ (Mann-Whitney *U* test).

compared to those who became pregnant (fold increase = 1.44 ± 0.16, $P < .05$; Figure 1).

Partial correlation analysis adjusting for age, time of infertility, endometrial thickness, and serum AMH levels showed an independent association between the implantation rate and the endometrial expression of α-inhibin ($r = -.391$, $P = .004$) and β-glycan ($r = -.248$, $P = .048$).

As shown in Figure 2, both α-inhibin and β-glycan gene expression levels were moderately accurate as predictive markers of endometrial receptivity. The area under the ROC curve was 0.692 (95% CI: 0.551-0.833) for α-inhibin and 0.678 (95% CI: 0.526-0.830) for β-glycan. The finding of $2^{-\Delta Ct} > 1.26$ for α-inhibin predicted implantation failure with a sensitivity of 0.64 and a positive likelihood ratio of 2.02, whereas the finding of $2^{-\Delta Ct} > 1.22$ for β-glycan predicted implantation failure with a sensitivity of 0.67 and a positive likelihood ratio of 2.53 (Table 3). The positive likelihood ratios >2 for both markers were interpreted as compatible with small but sometimes important shifts in probability.[19]

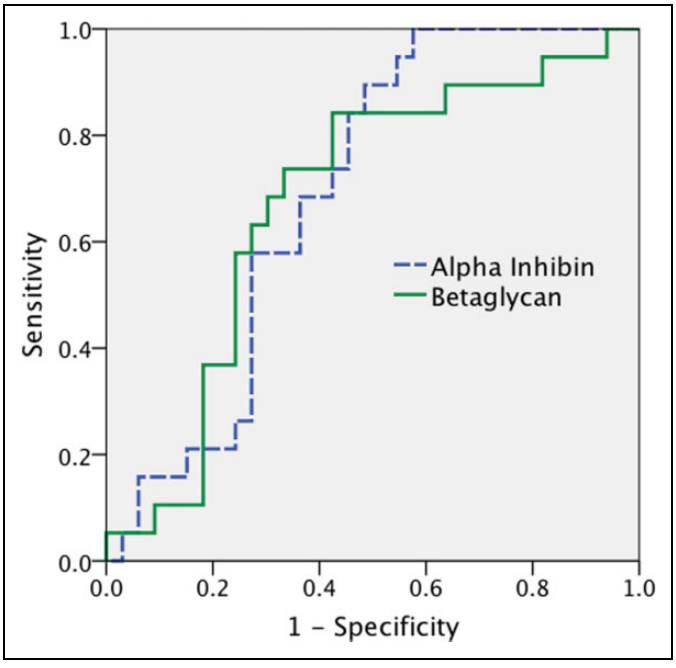

Figure 2. Receiver operating characteristic (ROC) curves for the prediction of implantation failure based on the endometrial expression of α-inhibin and β-glycan.

Table 3. Accuracy Measures (With 95% Confidence Intervals) of Secretory Phase Endometrial Expression of α-Inhibin and β-glycan mRNAs to Predict Implantation Failure in IVF With Fresh Embryo Transfer.[a]

	α-Inhibin	β-Glycan
Cutoff ($2^{-\Delta Ct}$)	>1.26	>1.22
Sensitivity	0.64 (0.47-0.78)	0.67 (0.50-0.80)
Specificity	0.68 (0.46-0.85)	0.74 (0.51-0.88)
Positive likelihood ratio	2.02 (0.99-4.10)	2.53 (1.15-5.58)
Negative likelihood ratio	0.53 (0.31-0.92)	0.45 (0.26-0.79)
Area under the ROC curve	0.692 (0.551-0.833)	0.678 (0.526-0.830)

Abbreviations: IVF, in vitro fertilization; mRNA, messenger RNA; ROC, receiver operating characteristic curve.
[a]The cutoffs are relative gene expression levels, normalized by the housekeeping gene *S26*.

Discussion

Members of TGF-β superfamily are widely expressed in the endometrium and have active roles in the implantation process.[20] A review by Singh et al[1] indicates that members of the TGF-β superfamily play a major role in implantation in mice and humans, and their deregulated expression and action could lead to absolute or partial failure of embryo implantation. The present study was conducted to determine the levels of endometrial α-inhibin and β-glycan gene expression according to the occurrence of clinical pregnancy in IVF cycles with fresh embryo transfer. We found that implantation failure was associated with a higher gene expression of α-inhibin and β-glycan

in the mid-luteal phase endometrium immediately before controlled ovarian stimulation for IVF.

It is still unknown why the patients with implantation failure expressed more α-inhibin and β-glycan than the control group. These molecular alterations might be either a cause or a consequence of failed endometrial differentiation. We can postulate, yet pending evidence of a causal relationship, that the enhanced presence of these molecules would block the effects of activin A on endometrial decidualization,[14] and the endometrium would become less receptive to embryo implantation.

Transferring good quality embryos does not guarantee implantation success, once the implantation rate is between 40% and 60% in IVF/ICSI procedures.[21] Human embryo implantation involves a synchronized cross talk between a receptive endometrium and a functional embryo and a complex sequence of signaling events, consisting in the acquisition of adhesion ligands together with the loss of inhibitory components, which are crucial to the establishment of pregnancy.[3] Endometrial biopsy can be used to identify a large number of molecular mediators to predict the success of embryo implantation, including adhesion molecules, hormones, cytokines, growth factors, and others.[22] Gene expression evaluation of human endometrium in fertile women or in patients with recurrent implantation failure has been combined with a bioinformatics approach to predict endometrial receptivity in the clinical setting.[23] This technology is promising, but it is also intrinsically limited by the interindividual and intercycle variability of endometrial transcriptome and proteome,[24] thus justifying novel markers to be discovered and evaluated, hopefully to improve the molecular portrait of a "receptive" endometrium.

The present results for α-inhibin and β-glycan are based on a quantitative gene expression assessment performed by RT-PCR. Diedrich et al[25] proposed that gene expression would be a better indicator for endometrial dating than histology, whereas Evans et al[26] noted that mRNA expression had a close correlation with endocrine markers, determining the nonreceptive and potentially receptive phases of endometrium. The differential expression of α-inhibin (2.1-fold) and β-glycan (1.4-fold) mRNA between our 2 study groups might be considered small had it been found unexpectedly in a multigene screening test. However, our hypothesis was based on the known mechanisms that imply activin signaling in fine-tuning endometrial physiology and support the link between endogenous activin antagonists and endometrial dysfunction.[7,9,14,27] Thus, from a Bayesian perspective, the relationship of these gene products with implantation failure has a fair pretest probability to exist and is reinforced by the present findings in a clinical model.

Although endometrial development in natural cycles may involve a different gene expression pattern compared to controlled ovarian hyperstimulation cycles,[28] the measurement of endometrial biomarkers could be useful to predict a lower chance to achieve pregnancy, providing a basis for patient counseling and for endometrial preparation and frozen–thawed embryo transfer.[29] Furthermore, if the present preliminary findings are confirmed in further studies with larger samples, as to allow the discrimination of patients with recurrent implantation failure, we can envisage the search for pharmacological tools to neutralize the effects of β-glycan and inhibin in the endometrium, increasing activin signaling and thereby improving endometrial decidualization and receptivity.

The results of the present study should be interpreted taking into account some methodological limitations. The functional implications of differential mRNA expression such as protein levels and protein signaling have not been evaluated here. Receptor levels and other activin-related molecules may also be involved in the cell response to altered inhibin and β-glycan levels.[4,7] Although the cyclic variations of α-inhibin and β-glycan in human endometrium have been well described,[8,9] their intercycle variability should also be investigated, which is important to establish the validity of assessing these markers in a menstrual cycle before that of embryo transfer. Our endometrial samples were collected between the 18th and the 20th day of the menstrual cycle on the month before treatment, therefore, the present results cannot be generalized to samples obtained at other menstrual cycle phases or during the treatment cycle. Because case–control studies do not allow calculation of prevalence-based measures, such as the positive and negative predictive values,[30] the predictive value of these molecules remains to be evaluated, preferably in association with other markers to improve the test accuracy and better estimate the risk of implantation failure.

In conclusion, the present data suggest that high expression levels of α-inhibin and β-glycan transcripts in secretory phase endometrium are associated with failure to achieve a clinical pregnancy after IVF and fresh embryo transfer.

Author Contributions

Camila O. Silveira and Carolina P. Rezende are equal contributors.

Declaration of Conflicting Interests

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