

Progesterone Receptor Phosphorylation is Associated to Claudin 1 and 6 Expression and Pregnancy Success in ART-Treated Women

Abstract

The success in infertility hormonal treatments and achieving a successful pregnancy is of great importance among infertile couples. Consequently the need to define new endometrial markers to evaluate the real impact of treatments is pressing. Since blastocyst implantation in a functional endometrium in the so-called "window of receptivity" is strongly associated to progesterone, our aim was to correlate the level of expression of progesterone-dependent claudins-1, -2, -3, -4, -5 and -6, $\alpha\beta 3$ integrin, LIF and progesterone receptor with pregnancy success in endometrial biopsies of primary infertile women. Endometrium was obtained during the secretory phase of the menstrual cycle from eight healthy and fertile women and 48 primary infertile women aged 30-47 years old being treated in the Infertility Clinic, Hospital "Angeles" del Pedregal, México.

Immunohistochemical analysis of the biopsies was performed with monoclonal and polyclonal antibodies reactive to claudins-1 to -6, LIF, $\alpha\beta 3$ integrin, progesterone receptor (PR) and its phosphorylated form (pPR). Sera obtained the same day of the biopsy were used to determine anti-Müllerian hormone concentration. The results showed that less claudin-1 and -6, LIF and integrin $\alpha\beta 3$ abundance together with pPR was associated with successful pregnancy in 20 of the women, the remaining 28 did not become pregnant but interestingly pPR could not be detected. In conclusion the examination of claudin-1 and -6 together with the phosphorylation of PR in the endometrial biopsies of infertile women under ART protocols will help predict greater successful pregnancy.

Keywords: Claudin; Integral LIF; Infertility; Endometrial

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Antonio de Jesús Paz-Martínez¹, Cecilia Elena Moreno-Barragán, Héctor Godoy-Morales², José Manuel Lozano-Sánchez², Emma Rodríguez³, Teresa Imelda Fortoul¹, Luis Felipe Montaña and Erika Patricia Rendón-Huerta^{1*}

¹Department of Immunobiology Laboratory Cell and Tissue, UNAM, México

²Department of Reproductive Biology, Hospital, México

³Department of Physiology, México

***Corresponding author:** Erika Patricia Rendón-Huerta, Department of Cell Biology and Tissue, Faculty of Medicine, U.N.A.M, Mexico, Tel: (011)55-56232191; Fax: (011) 55-56232399; Email: erendon@bq.unam.mx

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Abbreviations: BMI: Body Mass Index; PR: progesterone Receptor; pPR: Phosphorylated Form; ART: Assisted Reproductive Techniques; PBS Phosphate-Buffered Saline; DPP: Detecting Dots Per Point; BMI: Body Mass Index

Introduction

The prevalence of infertility in Western countries couples is close to the 15% figure [1]. Besides the classical causes of infertility: endometriosis, polycystic ovary syndrome, tubal patency failure and male infertility [2,3] a wide array of disorders associated with endometrial receptivity and thus ineffective blastocyst implantation, are being considered

Blastocyst implantation requires a physiologically functional endometrium where cell-cell adhesion molecules and tight junction proteins, expressed in the epithelium, establish cell polarity and regulate epithelial differentiation [4] so endometrial differentiation and gland development can be achieved [5]. Tight junction proteins are clustered at cell-cell contact sites and form a complex network in association with cytoskeleton and cytoplasmic proteins. They maintain the epithelial phenotype but also strengthen cell-cell adhesion, transfer signals between cells and serve as a physical barrier that regulate paracellular permeability [6,7]. The main component of tight junctions is claudins that form the paracellular tight junction seal in epithelial tissues [8]. Normal endometrium expresses claudin-1, -3, -4 and -5 [9] and its expression is regulated by progesterone

[10]. Claudin-4 is considered a mediator of embryo implantation since its mRNA increases during the early to mid-luteal transition [11] but it has not been associated with infertility.

Infertility is associated to disrupt implantation-associated $\alpha\beta 3$ integrin gene [3] and deficient endometrial expression of leukemia inhibitory factor, a cytokine released by uterine natural killer cells [12]. Endometrial claudins, $\alpha\beta 3$ integrin-related gene and leukemia inhibitory factor, are regulated by progesterone [13-16]. Therefore it is clear that embryo implantation in the so-called "window of receptivity" is strongly associated to progesterone and progesterone-dependent molecules [17,18]. Antimüllerian hormone concentration is considered as a "marker" for successful egg implantation as it is positively correlated with number of eggs retrieved but its value for predicting pregnancy after any given treatment cycle is poor. The success of the implantation process is also partially-dependent on the thickness of the endometrial [19] and sildenafil citrate has been successfully used to increase endometrial thickness [20].

The aim of our work was to correlate the establishment of pregnancy with the level of expression of claudins-1, -2, -3, -4, -5 and -6, $\alpha\beta 3$ integrin and LIF in endometrial biopsies obtained during the "window of receptivity" of primary infertile women being treated with sildenafil citrate and in vitro fertilization techniques.

Materials and Methods

Patients and tissue collection

Patients and controls were women aged 19-48 years old attending the Infertility Clinic at the Hospital Angeles del Pedregal, México. A comprehensive medical history and physical assessment was performed for each patient. Their fertility status was determined by a successful pregnancy one or two years previous to the biopsy. Control endometrium tissue specimens were obtained from eight healthy and fertile women, during the secretory phase of the menstrual cycle, whereas those of the study group were obtained from 48 primary infertile women aged 30-47 years old. All the samples corresponded to endometria from the midsecretory phase (cycle day 17 and endometrium width of 10-12 mm as determined by echocardiogram). All women from the study group were subjected to the Clinic' ovarian stimulation protocol and assisted reproductive techniques (ART) one month previous to embryo transfer.

Briefly, women received endometrial preparation for at least two cycles of estradiol 2 mg every 12 hs orally for 13 days. If the width of the endometrium was below 10 mm the dose increased to 2 mg every 6 hs. All the patients received aspirin 100 mg per day, folic acid 4 mg per day and sildenafil citrate (Tadalafil) 10 mg every 72 hs, during the cycle. Four days previous to the embryo-transfer all the patients received 400 mg of progesterone twice a day. At the end of these cycles, if the patient failed to get pregnant, as determined by a negative quantitative pregnancy test (serum b-hCG < 1.2 UI/ml) 12 days after embryo transfer, a Mock cycle was performed. In the latter cycle an endometrium biopsy was taken on day 17th of the cycle before in vitro fertilization cycle or cycles was performed. An experienced gynecologist obtained the endometria tissue specimens and an experienced histopathologist dated the tissue sample. All tissue samples of this case-control study were obtained with the informed consent of the patients. All the women received embryos previously screened by blastomeric analysis before implantation [21]. The Research and Ethics Committee of the Hospital Angeles del Pedregal, México, approved the study, which was conducted in accordance with international laws on procedures for human tissue handling of Helsinki declaration 1964.

Immunohistochemistry

Endometrial tissue specimens were fixed in neutral-buffered 4% ρ -formaldehyde at 4°C overnight and were subsequently paraffin embedded. Before performing immunohistochemistry, sections of the tissues were stained with hematoxylin-eosin to corroborate the usefulness of the biopsy. Serial sections, 4- μ m thick, were used for immunohistochemistry. Paraffin-embedded sections were dewaxed and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was achieved by boiling the samples in 0.01M Citrate of Sodium, pH 6 for 2 min. The quenching of endogenous peroxidase was achieved by incubation with 0.3% hydrogen peroxide in distilled water for 30 min at room temperature. After washing with phosphate-buffered saline (PBS), the slides were incubated with 2% albumin solution for 2 hr at room temperature and extensively washed with PBS. The slides were incubated at 4°C in a humid chamber overnight with primary antibody diluted in Tris-buffered saline containing 1% bovine serum albumin.

The antibodies were Rabbit polyclonal anti-LIF and anti-claudin 1 (#GTX81609 and #GTX54539, respectively, dilution 1:50, Gene Tex, Inc. Irvine, CA), anti-claudin 2 and anti-claudin-5 (#51-6100 and #34-1600, dilution 1:50 and 1:75 respectively, Invitrogen, Camarillo, CA) and anti-phospho-progesterone receptor (Ser 190)(#3171, dilution 1:750, Cell Signaling Technology, Inc); Rabbit monoclonal anti-progesterone receptor A/B (#8757, dilution 1:500, Cell Signaling Technology, Inc.); Goat anti-claudin 3, anti-claudin 4, anti-claudin 6 (#sc-17660, #sc-17664 and #sc-17669, respectively, dilution 1:200, Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-integrin $\alpha\beta 3$ (#ab78289, dilution 1:150, Abcam, Cambridge, MA).

At the end of the incubation period, the samples were extensively washed in PBS and labeled with a second biotin-labeled specific antibody system for 2 h (dilution 1:200) (LSAB* System-HRP kit (DAKO North America Inc. Carpinteria, CA)), followed by a 30 min incubation with peroxidase-labeled streptavidin, also included in the DAKO kit. At the end and after extensive washing the reaction was revealed with DAB for 2 min, washed with PBS/Tween-20 and nuclei were counterstained with CAT Hematoxylin (Bio Care Med., Concord, CA). The slides were mounted using Golden Bridge Mount (#E01-100, GBI Labs, Bothell, WA) at room temperature and evaluated with a Nikon Eclipse E600 microscope equipped with a photographic camera. Photographs from three different fields per slide were taken and the number of positive cells was determined. The intensity of staining was determined by detecting dots per point (dpp) in the photographs using the image analysis software Image J. All the data was compiled and a database sheet was constructed. All the antibodies were initially titrated to determine the optimal working dilution.

Anti-mullerian hormone determination

The day the endometrial tissue specimen was obtained a 5 ml blood sample was also obtained with a vacutainer tube. After clotting for 30 min, the tubes were spun and serum was immediately aliquoted and stored in -70°C freezers until use. A commercially available ELISA kit (Human MIS/AMH DuoSet, R&D Systems, MN, USA) was used to determine Anti-Mullerian hormone concentration. Normal values are in the 0.7-3-ng/ml ranges.

Statistical analysis

The database was analyzed with the JMP v10 software (SAS, Cary, NC). Univariate analysis was used to evaluate the possible relation between integrin expression and pregnancy whereas a multivariate analysis was used to evaluate possible correlations between claudin expression and final procedure outcome; the later results were corroborated with a Spearman's test. A p value < 0.05 was considered as statistical significant.

Results

The distinctive of the control and the study group is included in Table 1. The infertile group was subdivided into those reaching a pregnancy and those who did not. There were no differences in Body Mass Index (BMI) and age of menarche between the infertile women group that did and did not reach pregnancy, the only difference between the infertile women and the control group was the age.

The immunohistochemical analysis of gland and stromal cells of the endometrium biopsies of control women showed claudin-6 expression in 55% of them, claudin-5 in 21%, claudin-1 in 18% and claudin-2 in 4%. Interestingly, 48% of the cells were positive for LIF expression but only 7% of the cells were positive for integrin $\alpha\beta 3$ expression (Figure 1). Claudin-3 and -4 was expressed in 29 and 34% of the cells, respectively.

Compared to control biopsies and contrary to the above-mentioned results the percentage of cells expressing claudin-6 in the endometrial biopsies of the experimental women who became pregnant showed a highly significant decrease (17% vs 55%, $p < 0.01$); the decrease in claudin-1 expression was also significant (6% vs 18%, $p < 0.05$). The percentage of cells that expressed claudin-2, -3 and -4 was almost identical to control biopsies. There was a significant increase in the percentage of cells that expressed claudin-5 (32% vs 21%, $p < 0.05$). The percentage of cells expressing LIF and integrin $\alpha\beta 3$ was significantly lower for both proteins (19% vs 48% and 2% vs 7%, respectively; $p < 0.05$ for both) (Figure 1). Figure 2 shows a representative image of the immunohistochemical stain of claudins, integrin and LIF in infertile women.

The percentage of positive immunostained cells differed sharply in the endometrial biopsies of the experimental women who did not become pregnant. Claudin-1, claudin-2 and claudin-6 expression was significantly higher in comparison to those of women who became pregnant (23% vs 6%, 12% vs 4% and 36% vs 17%, respectively, $p < 0.01$ for all). Claudin-3,-4 and -5 as well as LIF expression was slightly higher but the difference did not reach statistical significance. Unexpectedly, the percentage of cells expressing integrin $\alpha\beta 3$ was significantly higher (17% vs 2%, $p < 0.01$) (Figure 2).

A multivariate analysis of the outcome of the reproductive technique procedure versus the endometrial expression of the evaluated proteins showed a positive correlation between integrin $\alpha\beta 3$ and claudin-5 expression ($p = 0.018$) and claudin-6 with claudin-1 ($p < 0.0003$) in the group that had a successful pregnancy. Integrin $\alpha\beta 3$ expression was significantly lower in non-pregnant women in comparison to pregnant women ($p < 0.05$). Other strong negative correlations in the non-pregnant group included claudin-6 vs claudin-5 ($p = 0.0063$), claudin-1 and LIF ($p = 0.0409$) and claudin-2 and LIF ($p = 0.0012$).

shows a representative image of the reactivity of the epithelia results. The final results of the ART procedure bear no relation to the epithelial reactivity. The great majority of biopsies showed immunostains that were considered as moderate. The serum concentration of anti-Mullerian hormone level is shown in Table1. The serum hormone concentration was independent of the epithelial reactivity in relation to the expression of claudins or the outcome of the ART procedure. The difference in value between the women who got pregnant and those who did not, reach statistical significance but interestingly the mean value of the women who got pregnant was below 1 ng/ml value. Progesterone receptor expression was clearly identified in almost all the nuclei of the glands and stromal epithelial cells in the endometrial biopsies of infertile women who got pregnant. The biopsies of those women who failed to get pregnant were also positive but the stain was appreciably less intense. Interestingly, the phosphorylated form of the progesterone receptor was only observed in the biopsies of women who got pregnant (Figure 2).

Table 1: Means and standard deviations of considered variables according to pregnancy status.

| | Pregnancy (n = 5) | No Pregnancy (n = 9) | P Value |
|-----------------|----------------------|-------------------------|--------------------|
| Age (years-old) | 40.2 (5.36) | 37 (4.27) | 0.289 |
| BMI (kg/m2) | 23.5 (2.84) | 24.8 (3.81) | 0.456 |
| Integrin (%) | 6 (8.94) | 37.78 (21.67) | 0.020 ^a |
| AMH (ng/ml) | 0.29 (0.29) | 1.92 (2.47) | 0.178 |
| Index | 6.32 (8.84) | 39.71 (21.03) | .001a |

a Correlation is significant at the 0.05 level (2-tailed).

Note: Values are expressed as mean (standard deviation); BMI: Body Mass Index; AMH: Anti Mullerian Hormone.

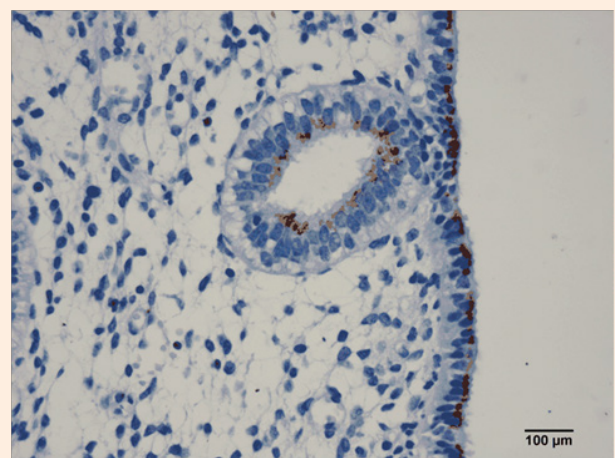


Figure1: Representative Image of Endometrial Integrin Avβ3 staining.

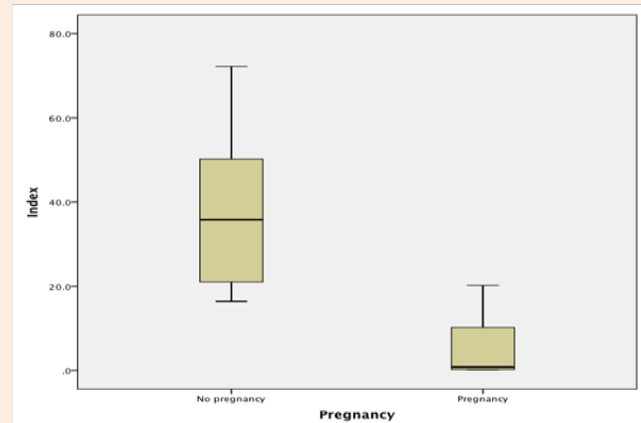


Figure 2: Box plots show median, interquartile range and extreme cases of individuals variables.

Discussion

Human blastocyst implantation is a complex process that includes apposition and attachment of the blastocyst to the receptive endometrium and invasion of the trophoblastic cells into the endometrium [22,23]. Different factors are involved in

fertility success. Embryonic implantation is tightly regulated and excess body weight with its associated increase in adipokines, is significantly involved in the decline in the natural fertility of mammals [24]. Our results showed that infertile women who did not get pregnant had a slight tendency to being overweight but the difference with the women who got pregnant and the controls was not statistically significant. Age is also a concern. Women in her late 30s and early 40s are at some disadvantage in terms of conception delay, ability to carry a chromosomally normal fetus until term and risk of trisomy conception [25]. Our results showed that all the infertile women were in their late 30s but there were no differences in age-range between those who got pregnant and those who did not; the possible reasons include normal zona pellucida thickness of embryos [26] or normal activation of the hedgehog signaling pathway [27] or simply that many of the maternal regulations which are impaired by age [28] were not affected. Antimüllerian hormone is positively correlated with number of eggs retrieved but its value for predicting pregnancy after any given treatment cycle is poor; low levels of AMH still can achieve reasonable treatment outcomes [29]. Antimüllerian hormone concentration in the control and the study group was within normal values considering the age range [30].

Embryonic implantation is also associated with modifications in the endometrial expression of claudins in the different phases of the menstrual cycle but mainly at the peak of the “transplantation window” [9,31,32]. The expression of claudin-1 and -5 is very low during the proliferative phase of a normal cycle in comparison with the secretory phase, but claudins-2, -3, -4 and -7 expression is almost identical [32] despite the fact that claudin-4 acts as molecular mediator of embryo implantation [33]. Interestingly, progesterone, a key hormone in the “transplantation window” has been shown to induce claudin-1, -3, -4 and -7 expression in normal human endometrial epithelial cells and to down regulate the barrier function during the menstrual cycle. Our results in infertile women who became pregnant after the treatment showed a downregulated expression of claudin-1, -2 and 6, integrin $\alpha\beta3$ and LIF in comparison with the endometrial biopsies of the infertile women who did not become pregnant. The down-regulation of claudins is clearly associated to modifications in ion and water interchange [34] and it has been recently shown that steroid hormones-regulated ion/water channel proteins are important for embryo implantation [35]. The expression of integrin $\alpha\beta3$ was lower in women who became pregnant in comparison with those that did not. This result was somehow surprising but the crucial issue is ¿what is considered as an adequate integrin $\alpha\beta3$ expressions? [36] Interestingly the expression of LIF was also downregulated in infertile women who became pregnant. LIF might influence blastocyst attachment through STAT3 activation and expression of integrins [37] but the expression of LIF is maximal only in decidualized stromal cells as a response to progesterone. Our study was performed in endometrial biopsies obtained one month prior to blastocyst implantation; therefore it is possible that the low integrin and LIF expression is representative of a normal endometrium. It is well known that LIF is produced at low levels in the endometrial stroma during the secretory phase of the menstrual cycle [38].

Our results strongly suggest that an increased expression of integrin $\alpha\beta3$ together with “normal” LIF expression in infertile women represent an abnormal endometrium.

The statistical analysis demonstrated a positive correlation between the decrease in integrin $\alpha\beta3$ with increased claudin-5 expression and pregnancy. Our results also showed that claudin-5 expression was enhanced in infertile women who got pregnant. Claudin-5 is a critical component of endothelial tight junctions that control pericellular permeability. Estrogen diminishes claudin-5 expression and consequently the uterine cell permeability increases [39] contributing to endometrial edema after the estrogen stimulation. Since Sildenafil citrate is known to improve the high radial artery-resistance index and endometrial thickness and prevent the increase of vascular permeability [40]. It is highly possible that the increase in claudin-5 expression we observed in the endometrial biopsies of women who got pregnant is secondary to the sildenafil citrate treatment. Such an effect would mimic a “normal” endometrium paracellular milieu.

The expression of the molecules that we evaluated is regulated by progesterone [13-16]. Interestingly, our results showed that infertile women who got pregnant overexpress progesterone receptor, in comparison with control biopsies and its phosphorylated form, thus priming the receptor for robust transcriptional activation in response to ligand [41]. Endometrial biopsies from women who did not get pregnant expressed much less progesterone receptor, in comparison with control biopsies and failed to express the phosphorylated form of the receptor. The phosphorylation of the PR is secondary to binding with its ligand. It is possible that the amount of progesterone receptor in the infertile women who did not get pregnant is low because abnormal estrogen levels. It is also possible that the receptor is functionally and/or biologically abnormal [42,43].

In summary, our results show that successful embryo implantation in infertile women undergoing ART is more directly related to the biological efficiency of the progesterone receptor that is crucial to support the over- or under-expression of claudin 5, LIF and integrin $\alpha\beta3$ expression in the endometrial tissue. The expression of these molecules in the endometrium is essential for embryo implantation. Our results also state the need to define new endometrial markers to evaluate the real impact of hormonal treatments on pregnancy rates. The molecular definition of a “normal and receptive endometrium” is far from being delineated.

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