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Effect of Controlled Ovarian Hyperstimulation on Temporal and Spacial Expression of Integrin- β3 Osteopontin in the Endometrial of Mouse

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Abstract

Objective: To explore the effect of controlled ovarian hyperstimulation (COH) on endometrial receptivity of mouse. Methods: Sexually matured Kunming Mice were randomized to a study group (COH group) and a control group. The endometrium in both groups was removed respectively on pregnant day (PD) 0, 2, 4, 6 and 8 for detection of the temporal sequence of the mRNA expression of integrin-β3 and its ligand osteopontin (OPN) in the endometrium by using in situ hybridization method.

Results: (1) On the second day of pregnancy, the endometrial integrin-β3 mRNA expression in the study group was significantly higher than that in the control group ($P<0.01$), while on pregnant day 4 and 6, it is much lower in the study group ($P<0.05$). There was even no significant difference ($P>0.05$) when it comes to pregnant day 8 (PD8) between two groups. (2) The ligand osteopontin mRNA expression on PD 4, 6 and 8 in the study group were significantly lower than that in the control group ($P<0.01$).

Conclusion: Controlled ovarian hyperstimulation may affect the endometrial receptivity by interfering with the time sequence of integrin-β3 and its ligand osteopontin's mRNA expression, bringing forward the peak of endometrial integrin-β3 expression and destroying the intrinsic coordinative relationship between integrin-β3 and its ligand osteopontin.

Keywords: Contolled ovarian hyperstimulation; Endometrial receptivity; Integrin-β3; Osteopontin; Temporal and special expression

Introduction

As we all know, a high quality embryo and a favourable endometrial environment for implantation are key points in the successful process of the assisted reproductive technology (ART). The application of controlled ovarian hyperstimulation (COH) technique ensures multiple simultaneous mature follicles, making it possible to select much more highly-qualitied embryos for transplantation after fertilization. With increased availability of follicles, the rate of fertilization could reach 75-90%, while implantation and pregnancy rate still remain at a low level of 30-40%. Therefore, the effect of superovulation on the endometrial implantation window has aroused more and more attention in the field of reproductive medicine. Integrin-β3 participates in embryo implantation by binding with its ligand osteopontin (OPN) in an arginine-glycine-aspartate (Arg-Gly-Asp,RGD)-dependent manner. In this study, we dynamically observed the expression of mouse endometrial Integrin-β3 and its ligand OPN in the peri-implantation period during superovulation in an attempt to provide scientific clues for the study of effects of COH on endometrial receptivity.

Materials and Methods

Animals

Healthy, sexually mature and specific pathogen-free Kunming mice aged 8-10 weeks, weighing 40±5g and born in the same period were provided by the experimental animal center of Shandong University School of Medicine (Jinan, China). The estrous cycle was determined by vaginal secretion smear. Mice with two consecutive normal estrous cycles were raised for a week and then used for the experiment. Fifty female mice and fifty male mice in estrus were selected for the study (Quality Permit No.: SCXK (SD) 20030004).

Main reagents

a) The integrin-β3 of mouse in situ hybridization kit was purchased from Wuhan Boster Biological Technology Co., Ltd.

(Wuhan, China) using digoxin effectively labeled integrin-β3 oligonucleotide probes. The target gene mRNA sequences of integrin β3 are as follows:

a) 5'-GACACCTGTGAGAAGTGCCCCACCTGCCCA-3'

b) 5'-GGATGACTGTGTCGTCAGATTCCAGTACTA-3'

c) 5'-GCTAAATTTGAGGAAAGGCGCGCCAGAGCA-3'

b) The OPN protein in situ hybridization kit was purchased from Wuhan Boster Biological Technology Co., Ltd., using digoxin effectively labeled OPN oligonucleotide probes. The target gene mRNA sequences of mouse OPN are as follows:

① 5'-GACCCATCTCAGAAGCAGAATCTCTTAGCCCCACA-3'

② 5'-TCTGACGAGACTCACCATTCCGATGAATCTGATGA-3'

c) 4% poly-L-lysine, 0.1% DEPC-double distilled water, 3% citric acid, 20% glycerin, PBS, 2×SSC, 0.5×SSC, 0.2×SSC, and DAB used for in situ hybridization were all provided by Wuhan Boster Biological Technology Co., Ltd.

Grouping

The mice were randomized to two groups: the study group (COH group) and the control group, from each group five mice were selected respectively at day 0, 2, 4, 6 and 8 after being kept in the same cage, totaling 25 mice in each group.

Drug administration

In the study group, the animals were intraperitoneally injected with 12 IU human menopausal gonadotropin (hMG) at 6PM, and with 12IU human chorionic gonadotrophin (hCG) 48h later. Then they were kept in the same cage at a 1:1 M/F ratio, and the following day was regarded as pregnant day (PD)1. While in the control group, the day of noticing vaginal estrus was regarded as PD0, and the following morning after put in the same cage when the vaginal plug was noticed was regarded as PD1.

Sample Collection

At 10 AM of the designated days (PD 0, 2, 4, 6, 8), the mice were sacrificed by cervical vertebral dislocation. The uterus was removed by laparotomy and the endometrium was scraped for experiment. They were then fixed in 4% paraformaldehyde, paraffin-embedded and sliced. What calls for special attention was that in PD 4, 6 and 8 groups, curettage should include the embryo at the implantation site and the decidua with the help of a dissecting microscope. The expressions of integrin-β3 and its ligand OPN mRNA in the endometrium were detected by in situ hybridization at PD 0, 2, 4, 6 and 8 respectively.

Procedure of in Situ Hybridization

FiX the fresh mice endometrium promptly with fixing solution made up of 4% paraformaldehyde and 0.1M PBS （PH7.0~7.6containing 1/1000DEPC. Slicing waxstones into 6-8um thick after general dehydration, waxdipping,andembedding. Following exposing mRNA nucleic acid fragment,after-fixing, prehybridization, hybridization,washing after hybridization, zutropfening sealing fluid, biotinylated mouse anti-digoxin,SABC, biotinylated peroxidase,DAB coloration, alcohol dehydration,xylene hyalinization, coverslipping etc.

Observation of in Situ Hybridization Sections

Tissue sections without addition of integrin-β3 probes were used as negative control. Positive expression of integrin-β3 mRNA was symbolized by appearance of yellow-brown particles in cytoplasm: ＋＋＋ as strongly positive, ＋＋ as positive, and the absence of yellow-brown particles as negative. The slides were interpreted by two pathologists independently, and any disagreement between them was solved by slide review and consensus. OPN histological sections were handled by the same method. Each in situ hybridization section was observed under Nikon ECLIPSE-80i by randomly selecting two endometrial sites, and mean optic density (MOD) of each visual field was calculated with the IMAGE-PROPLUS6.0 image analysis system.

Statistical Analysis

Statistical analysis was performed by SPSS17.0. Measurement data were expressed as mean±SD（±Sand verified by t test (α=0.05 as the standard). Enumeration data were verified by χ2 test; when T<5, Fisher test was used for precision test. Bivariate correlation was analyzed by Pearson test.

Result

a) Histological location of integrin-β3 mRNA expression in the mouse endometrium during the peri-implantation period

b) Integrin-β3 mRNA expression in the mouse endometrium was highly identical in space during the peri-implantation period. It was mainly observed in the cavitary and glandular epithelial cytoplasm, and no significant expression was observed in the intimal mesenchyma.

c) Comparison of MOD in terms of temporal phase of integrin-β3 mRNA expression between the two groups

d) Histological location of OPN mRNA expression in the mouse endometrium during the peri-implantation period

e) OPN mRNA expression in the mouse endometrium was also highly identical in space during the peri-implantation period. It was observed mainly in the cavitary and glandular epithelial cells, and only weak slightly observed in the mesenchyma.

f) Comparison of MOD in terms of temporal phase of OPN mRNA expression between the two groups

Discussion

Laws of integrin-β3 and its ligand OPN mRNA expression in the mouse endometrium during the peri-implantation period

Mutual recognition between the uterine endometrium and embryonic cells is necessary for the establishment of pregnancy.

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In the process of embryo implantation, certain polypeptides secreted by the endometrial gland is really useful for the establishment and maintenance of pregnancy. Integrin-β3 is generally accepted as a specific molecule for evaluating receptivity of the uterine endometrium in the implantation period [1]. It is expressed in the endometrium after the middle luteal phase (usually after the 19th day of a normal menstrual period), occurring simultaneously with the opening of the endometrial implantation window. Integrin-β3 can recognize the extracellular stromal ligand of arginine-glycine-aspartic acid (RGD), whose sequence plays an important role in attachment and growth of trophocytes [2]. OPN is the substrate of adhesion molecules in intimal epithelial cells, in which integrin-β3 is expressed. After combined with integrin-β3 [3,4], they could transmit extracellular information into cells, causing change in the intracellular structure and at the same time triggering elevation of calcium ions, the second messenger in cells, which further activates protease and phosphatase to initiate extracellular signal transduction and transmit information to the nucleus to regulate gene expression, thus influencing cell proliferation, differentiation and behavior. In addition, integrin-β3 on blastocysts can also specifically bind with the intimal OPN by recognizing the structure of RGD [2], which plays an important role in the process of blastocyst implantation [5,6]. Some scholars have successfully detected high expression of OPN in the decidual intimal mesenchyma of pregnant mice, primates and humans, believing that it is a gene marker of intimal decidual change [7,8], which indicated than the coordinative expression of integrin-β3 and its ligand OPN may be closely associated with decidual change of the endometrium. Other studies also found that OPN and integrin-β3 were co-expressed in intimal glandular epithelial cells, decidual mesenchymal cells, and the blastcyst trophoblasts invading the endometrium during the peri-implantation window of blastocytes in baboons, mice and rabbits. Lessey proposed a "sandwich" blastocyst implantation model: using integrin-β3 expressing on the blastocyst and endometrial surface as the receptor of OPN, after combined with OPN it could promote the attachment of blastocyst onto the endometrium [9,10]. During the implantation window period, the expression and affinity of endometrial integrin is remarkably increased due to the regulation of steroids and a series of cytokines. As a result, the endometrium reaches its greatest receptivity. Integrin-β3 also expresses on the surface of blastcyst trophoblast cells. In both cases, integrin-β3 binds with OPN, forming a recognition compound: OPN is located between the two molecules of integrin and mediates the mutual connection between the blastocyst and the endometrium. To reach this satisfied condition ready for pregnancy, the greatest receptivity of the endometrium and the simultaneous growth of the blastocyst are two prerequisite points. Though the integrin exists on the surface of cells in large groups, it is indeed weak in binding with the ligand. However, gradual accumulation of this weak binding ability can increase the total binding ability on the surface of the endometrium. By accumulating countless "sandwich" models, the endometrium reaches a status of blastocyst receptivity.

Effect of COH on temporal sequence of integrin-β3/ OPN protein expression

Our results showed that integrin-β3 mRNA expression in the COH group reached the peak on PD day 2, which was much earlier than that in the control group (PD2 vs PD4), while the peak value was significantly lower than that of the normal group (0.62±0.04 vs 0.79±0.04, $P<0.05$). On PD day 4, 6 and 8, integrin-β3 mRNA expression in the COH group was lower (0.54±0.09 vs 0.79±0.04, $P<0.01$; 0.52±0.04 vs 58±0.04, $P<0.05$; 0.51±0.03 vs 0.52±0.03, $P>0.05$, respectively). It indicated that superovulation probably affect the establishment of endometrial receptivity by reducing the expression level of integrin-β3 mRNA and binging forward the expression peak of it.

To the best of our knowledge, there is no report in the literature about the influence of superovulation on OPN expression, and about the regime monitoring on the expression of integrin-β3 and its ligand OPN in the window period of endometrium. Our study showed that OPN expression in the study group was not significantly different from that in the normal group on PD 0 (0.15±0.02 vs 0.15±0.03, $P>0.05$); insignificantly lower on PD 2 (0.34±0.06 vs 0.39±0.03, $P>0.05$); and significantly lower on PD 4, 6 and 8 (0.88±0.07 vs 1.08±0.06, 0.56±0.05 vs 0.90±0.05, 0.60±0.02 vs 0.88±0.04, $P<0.01$, respectively). OPN monomer binds with integrin-β3 via RGD sequence,and combines with embryonic trophocytes via non RGD sequence; or OPN works as a bridging molecule to connect the uterus and the blastocyst in the form of polymer which is bind with both sides via RGD sequence. It is therefore presumable that COH probably interferes with normal embryo implantation by affecting the expression of OPN. This finding provides scientific evidence for further research of influence of superovulation on endometrial receptivity.

Conclusion

Our study indicates that COH treatment may decrease the clinical pregnancy rate by affecting the temporal and spacial expression of integrin-β3 and its ligand OPN in the endometrium, thus interfering with the establishment of normal implantation. Nevertheless, as embryo implantation is a complex process and could be influenced by multiple factors, it can hardly be elucidated by any single index or approach. How to give an objective evaluation on the influence of COH on receptivity of the uterine endometrium would be the focus of future research. In addition, how to use superovulation medications rationally in assisted with reproduction, how to adopt individualized therapeutic regimens, and how to minimize the adverse influence of superovulation by using minimal stimulation protocols and natural cycle protocols may be the trend of future development.

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