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Original Article

Effects of COH on the expression of connexin43 in endometrial stromal cells

Guolin Liu ^{a, b}, Yuanyuan Tang ^b, Yibing Han ^b, Xiaoming Teng ^{b, *}^a People's Hospital of Fu Yang, No. 63 Lu ci Street, Yingzhou District, Fu Yang, Anhui Province, China^b Shanghai First Maternity and Infant Hospital, Tongji University, NO. 2699 Gaoke West Road, Pudong New Area, Shanghai, China

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ABSTRACT

Objective: The purpose of this study was to investigate the effect of controlled ovarian hyperstimulation (COH) on gap junction, and to induce the effect of an estrogen level overdose on gap junction *in vitro* by COH. Here, we mainly focus on connexin43 (Cx43), progesterone receptor (PR) and prolactin-related protein (PRP), and CyclinD3 genes expression, as well as the expression of Cx43 protein, were investigated.

Materials and methods: Mature BDF-1 mice were divided into different COH, and the mouse uterus was isolated, Paraffin sections evaluate the effect of COH on mouse uterine endometrial morphology. The other part was used for the extraction of mouse uterine endometrial stromal cells (ESC), some related gene changes are detected. Human ESC were isolated from human endometrium by primary culture, the estrogen concentrations 10^{-6} mol/L, 10^{-7} mol/L were added, the changes of Cx43 gene and related proteins were detected, too.

Results: (1) HE staining showed that in the ovulatory endometrium of mice in the high super ovulation group, uterine glands in the stromal layer were significantly increased, the relative vascular tissues was less abundant. (2) In three groups of COH mice, the expression of Cx43, PR, and PRP genes in ESC was significantly different ($P < 0.05$). (3) *In vitro* ESC in the COH group showed significant differences in Cx43, PR, and CyclinD3 gene expression ($P < 0.05$), and showed an obvious dose effect. In addition, Western blot analysis showed that the Cx43 protein and Cx43 gene expression were similar.

Conclusions: (1) Animal experiments study showed that Cx43 gene expression in ESC was significantly decreased in hyper COH, in addition, the advance in gene expression was significantly earlier, suggesting decidualization appeared significantly earlier. (2) *In vitro* COH demonstrated when the estrogen concentration used was higher, the expression level of Cx43 gene and protein was lower. Combined with animal experiments, the endometrium decidualization was advanced in mice that were underwent hyper COH, which may reflect the endometrial receptivity.

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Introduction

In the treatment of *in vitro* fertilization and embryo transfer (IVF-ET), it is the key to obtain eggs. In COH, the use of excessive gonadotropin agonist and gonadotropin stimulation interferes with normal hormone levels in the body. Under the influence of the super-physiological of hormone, the endometrium becomes changes and becomes different when compared to that of a natural

cycle [1]. At the same time, COH also changed the levels of estrogen and progesterone, resulting in the production of estrogen in the body, which may interfere with receptivity of the endometrium and affects normal implantation of embryo. The implantation rate of embryos was reduced after different COH protocols, however the exact mechanism involved remains to be elucidated [2].

In the process of COH, follicle formation and maturation are affected not only by exogenous hormones, but also by the signals transmitted by their own secreting hormones. These complex signaling processes need to be accomplished through gap junction protein-mediated intercellular communication channels [3]. Gap junctions (GJ) are widely distributed and cover almost all animal tissues. GJ are transmembrane channels between adjacent cells. Gap junction intercellular communication (GJIC) can regulate

* Corresponding author. NO. 2699 Gao ke West Road, Pudong New Area, Shanghai, China.

E-mail addresses: liuguolin2013090@163.com (G. Liu), 89608162@qq.com (Y. Tang), ybhan1967@126.com (Y. Han), tengxiaoming@hotmail.com (X. Teng).

various physiological processes, and Connexin (Cx), the basic structural and functional protein that constitutes GJ, is located on adjacent cell membranes. Each of the six transmembrane protein subunits is arranged around the central pore to form a connector. The channel structure formed by the docking of multiple connectors are called GJ [4].

GJ play a key role in cell growth, differentiation, vascular remodeling, tumorification, and apoptosis, and are important players in many diseases, such as cancer and endometriosis. In previous studies, it has been shown that gap junctional communication in ESC regulates endometrial decidualization and angiogenesis. At present, more than 20 types of gap junction family proteins have been identified, among them, connexin43 (Cx43) is the most important gap junction protein. In previous studies, it has been shown that Cx43 is widely expressed in endometrial tissues [5].

Mice bearing a Cx43 gene mutation showed a decrease in both decidual angiogenesis and placental formation [6]. In studies that focused on mouse embryonic stem cells, it has been shown that deletion of the Cx43 gene resulted in the disruption of gap junctional communication, leading to a significant decrease in neo-vascularization during decidualization of the endometrium that resulted in the cessation of embryo development and loss of early pregnancy [7]. Therefore, the purpose of our study was to study the effect of COH on GJ, and to simulate the effect of over dose estrogen levels on GJ *in vitro* by COH.

Materials and methods

Mice and endometrial tissue

Sexually mature female BDF-1 mice (provided by experimental animal center of Tong ji University) were selected. The average body weight of mice was 25–30 g at the age of 6–8 weeks. Mice had access to food and water *ad libitum*. The illumination time was 12 h and the night time was controlled at 12 h. A total of 30 BDF-1 mice were randomly divided into a control group, normal control ovulation group, and hyper overstimulated ovulation group (n = 10 rats per group). At 6 pm, normal saline was injected intraperitoneally in mice in the control group, 10 IU PMSG was injected intraperitoneally in mice in the normal controlled ovulation supernatant group. Mice in the ovulatory hyperstimulation group received 30 IU PMSG 0.06 ml intraperitoneally. After 48 h, mice in each group were injected with 10 IU hCG 0.06 ml each at 6 pm. After 14 h, mice were euthanized, the uterus of each mouse was harvested. Endometrial sections were prepared, and the morphological comparison was performed. Another part of the mouse uterus was used for the isolation and extraction of mouse ESC.

Human endometrial tissue was obtained from the Shanghai first maternity and infant hospital (Shanghai, China), and the study was approved by the Tong ji University ethics committee, and informed consent was given by every patient. The endometrium in hysteroscopic hydrotubation when scraping patients collected a total of five patients with regular ovulation endometrium. Endometrial tissue was obtained in the proliferation of early, all patients in the 3 months prior to surgery without hormone therapy, age less than 35 years of age, and exclusion of endometriosis, adenomyosis, myoma of uterus, ovarian cancer, chronic pelvic pain, and other pelvic diseases.

ESC collection and culture

(1) Immediately after collection (<2 h), endometrial tissue was rinsed with sterile saline and placed in DMEM/F12 medium, containing streptomycin. (2) Endometrial tissue was placed into

35 mm petri dishes, then cut into about 1 mm³ pieces. Next, collagenase IV (final concentration 1 mg/ml) was added and the dish was transferred to a 37° incubator for 1 h (3) The supernatant was collected for 700 rpm and centrifuged for 7 min. After that, the supernatant was discarded and the cells were resuspended. Then, cells were well mixed, and a 200-targets (aperture 74 μm) cell filter was connected to a 5 ml sterile syringe for filtering. (4) To collect the cell filtrate, cells were centrifuged at 1000 rpm for 7 min in room temperature, the supernatant was discard, and the cells were resuspended in an appropriate amount of culture medium, and mixed it repeatedly. (5) Cells were inoculated in the appropriate amount in the culture dish. After ~30–60 min, the cells were observed to check for adherence to the culture dish. (6) After 12 h, the cells were changed gently and cultured continuously. The growth of cells was observed under a microscope. At 90% confluency, cells were subcultured. (7) The estrogen concentrations were 10⁻⁶ mol/L, 10⁻⁷ mol/L, respectively for the control group (C group), 10⁻⁶ mol/L group (named E6 group), 10⁻⁷ mol/L group (named E7 group).

HE staining

The uterus of mice in the control group, normal COH and super COH were fixed with 4% paraformaldehyde for 24 h. Then, the uterus was rinsed with tap water for 24 h, after which the tissue was dehydrated in alcohol at the following concentrations and times: 70% alcohol for 30 min, 80% alcohol for 30 min, 90% alcohol for 30 min, 95% alcohol for 30 min, 95% alcohol for 30 min, 100% alcohol for 30 min, and 100% alcohol for 30 min. Then, tissues were placed in chloroform solution and allowed to stand at room temperature for 30 min; chloroform paraffin was placed in an incubator at 60° for 3 h, the dehydrated uterine tissue was immersed in paraffin I, paraffin II (all 60°), and the wax was soaked for 40–50 min respectively. Subsequently, uterus tissue was quickly embedded, and 5 μm sections were cut. Sections were placed on glass slides, which were disposed by APES in advance. Next, sections were fixed and baked at a temperature of 45 °C. Sections were first treated with dimethylbenzene dewaxing; dimethylbenzene I for at least 30 min, then with dimethylbenzene I for at least 30 min. Next, sections were hydrated with gradient alcohol as follows: 100% alcohol for 5 min, 95% alcohol for 5 min, 90% alcohol for 5 min, 80% alcohol for 5 min, 70% alcohol for 5 min, and double distilled water for 5 min. Sections were rinsed with running water for 10 min and dried. Then, sections were immersed in double distilled water, and soaked for 3 times, five minutes every time. Washed sections were drained on filter paper and incubated in hematoxylin dye solution for 3–5 min. Sections were flushed with running tap water for 10–30 s. Sections were immersed in hydrochloric acid alcohol. Water was kept running for 1–2 h. Sections were dried with filter paper, incubated in eosin solution for 10 min, and placed under the running water for about 10 min. Excessive eosin solution was removed. After dehydration, sections were dehydrated using the following alcohol gradient: 70% alcohol for 5 min, 80% alcohol for 5 min, 90% alcohol for 5 min, 95% alcohol for 5 min, 95% alcohol for 5 min, 100% alcohol for 5 min, 100% alcohol for 5 min, xylene solution for 30 min, xylene solution for 30 min. Sections were sealed with neutral gum, dried and preserved at 37 °C. Staining was observed using an Olympus microscope (JAPAN).

QPCR

Total RNA was isolated from ESC using 5X All-In-One RT MasterMix Kit (Sigma, St. Louis, MO, USA cat#12183025) following the manufacturer's protocols, and frozen at -80 °C until analyzed. cDNA was synthesized from mRNA samples and subsequently used

as a template for conventional, end-point PCR assays. Primers for PCR amplification were constructed by Integrated DNA Technology, Inc. and sequences are presented in Table 1. As a control, reverse transcriptase was excluded from the amplification mix (data not shown). PCR was performed in an Opticon thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: one denaturation cycle of 94 °C for 3 min, followed by 28 amplification cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. cDNA amplicons were visualized on 1.0% agarose gels containing 0.2 µg/µl ethidium bromide.

Western blot analysis

Western blot analyses were performed on whole-cell extracts that were obtained by vortexing ESC in cell extraction buffer, followed by protein determination using a bicinchoninic acid protein assay kit (Sigma Chemical Co, St. Louis, MO, USA) Cell lysates (50 µg of total protein) were separated on 4–12% SDS-PAGE gradient gels, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with 5% skim milk in phosphate-buffered saline (PBS), containing Tween-20 (PBST). Caspase 3 was detected using a rabbit monoclonal antibody (1:1000 dilution, cat# 9664; Cell Signaling Technology, Danvers, MA, USA). CX43 was detected using a rabbit polyclonal anti-CX43 antibody (1:1000 dilution, cat# 3512, Cell Signaling Technology, USA). Immune-reactive bands were visualized by incubation with a secondary goat anti-rabbit antibody (1:30000; cat# 31460, USA) conjugated to horseradish peroxidase (HRP), followed by an enhanced chemiluminescence (ECL) system reaction (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were washed, reprobbed with mouse monoclonal anti-human β-actin antibodies (1:1000 dilution, cat# 31430, Sigma, St. Louis, MO, USA), and developed in an identical manner to ensure even loading.

Statistical analysis

For statistical analysis, SPSS19.0 statistical software was used. Experimental data were expressed as the mean ± standard deviation. Single factor analysis of variance (ANOVA) was used to determine the differences between groups. Differences between two groups were compared by independent sample t test ($P < 0.05$).

Results

Effects of different control superovulation regimens on endometrial morphology in mice

A: ESC were isolated from the mouse uterus (Fig. 1A). B: No other tissues such as blood vessels and uterine glands were found.

Table 1
Primers used in this study.

Candidate gene	Sequences
CX43 (mouse and people)	sense (5'-TACCATGCGACCACTGGTGCCT) antisense (5'-GAATTCGGTTATCATCGGGAA)
PR mouse	sense (5'-AAAGGGGAGCTGGTCTCT) antisense (5'-TCACCTCTGGTGTCTCTTG)
PRP (mouse)	sense (5'-TTATGGGTGCATGGATCACTCC) antisense (5'-CCCACGTAAGGTCATCATGGATT)
CyclinD3 (human)	sense (5'-CTGGCCATGAACACTCTGGA) antisense (5'-GCCAGAAATCATGTGCAAT)
PR (human)	sense (5'-GCCACAATACAGCTTCGAG) antisense (5'-AGGGCTTGGCTTTCATTGG)
β-ACTIN	sense (5'-CATGTACGTGCTATCCAGGC) antisense (5'-CTCCTTAATGTACCCACGA)

However, the nuclear size of stromal cells varied (Fig. 1B, arrow-head). C: In the normal hyperovulation group, stromal cells began to differentiate into small vessels and uterine glands increased (Fig. 1C, arrowhead). D: In the endometrium of the high dose hyperovulation group, the uterine glands in the stromal layer increased significantly (Fig. 1D, arrowhead).

Identification of mouse and human ESC

Extracted mouse and human ESC specifically expressed the specific protein Vimetin protein of stromal cells, indicating that the extracted cells were ESC and expressed Cx43 protein (Fig. 2).

Comparison of expression level of Cx43, PR, and PRP mRNA in endometrial stromal cells

Real-time fluorescence quantitative analysis showed that there was a significant difference in the expression of PRP gene in ESC of three groups of controlled hyperovulation mice (Fig. 3, $P < 0.05$). In mice in the hyper-stimulation group, the expression of Cx43 was significantly inhibited, but the expression of PRP gene was increased significantly, and the time of PRP expression was advanced.

Expression of genes and proteins in human ESC treated with different hormones

After treatment of human ESC with different concentrations of estrogen, Cx43, PR, and CyclinD3 gene expression was significantly different between groups (Fig. 4, $P < 0.05$). In particular, high concentrations of estrogen significantly inhibited gene expression of Cx43, PR, and CyclinD3, whereas low concentrations of estrogen promoted Cx43, PR, and CyclinD3 gene expression. The effects were dose dependent. Together, Western blot analysis showed that Cx43 protein expression was consistent with Cx43 gene expression.

Discussions

The most satisfying outcome of *in vitro* fertilization and embryo transfer is that a sufficient number of eggs can be obtained without affecting the normal physiological status of the endometrium [8]. Under normal physiological conditions, all types of steroid hormones in women of childbearing age maintain a dynamic balance under the influence of hormones and many other influencing factors. A series of complex changes in the function and morphology of the endometrium allow for normal development of the embryo and the development of normal decidua of the endometrium. This is beneficial to implantation and further development of embryo. However, in assisted reproductive technology, the superovulation routine application of steroid hormones *in vivo* produce a super physiological dose, which interferes with endogenous steroid hormones, thereby losing the moderating effect on the physiology of the normal endometrium, a situation in which endometrial development and embryonic development cannot be coordinated.

In mice, the decidua process lasted for three days (4.5–7.5 days after fertilization), and the highly differentiated endometrium stromal cells were diploid or polyploid [9]. It was found that decidualization of mice in the high-dose superovulation group began on the second day, and was significantly advanced, resulting in embryonic development and endometrium that was unable to synchronize. In mice, the implantation area of the embryo is accompanied by decidualization of the ESC as a significant process of vascular remodeling, and the resulting vascular network supported proper growth and development of the implanted embryo [10,11]. In human beings, the process of vascular remodeling of the

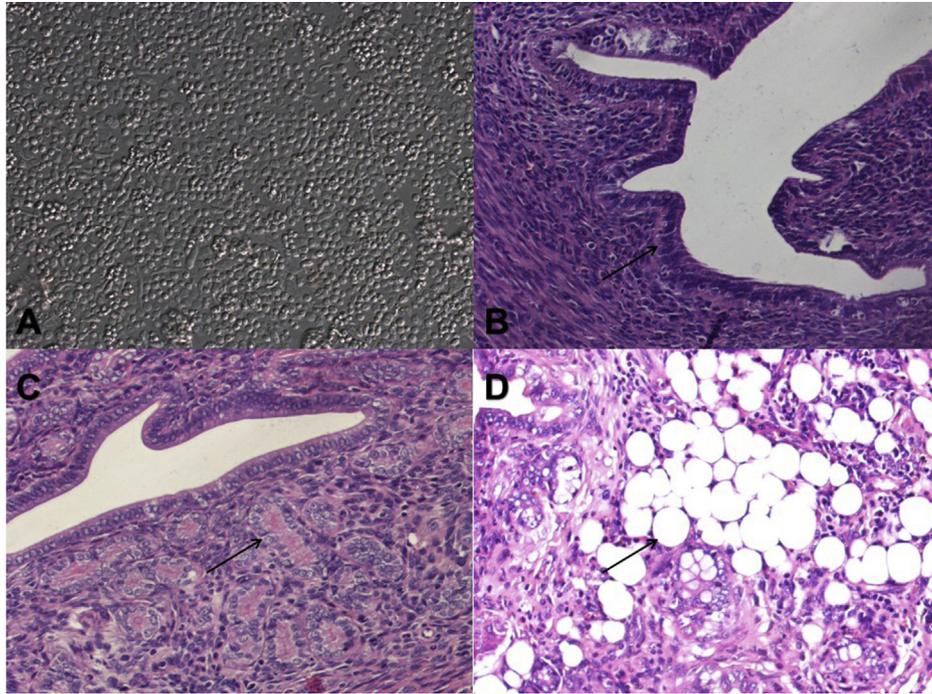


Fig. 1. Effects of different control superovulation regimens on endometrial morphology in mice: A: A is a normal isolated mouse endometrial stromal cell. B is endometrium of the control group, the arrowhead were showed that ESC were arranged 8–12 layers. C is normal hyperovulation group, the arrowhead were showed that red blood cells appearing in the vascular lumen. D is high dose hyperovulation group, the arrowhead indicates a decrease in vascular tissue, and a large amount of secretions entered the uterine cavity.

endometrium is a stage of every menstrual cycle. The close relationship between decidua and these vascular networks produces factors that regulate the growth of blood vessels. Recent studies have shown that Cx43 may affect the communication between decidual cells and blood vessels, as well as the process of decidualization [7]. ESC in pregnant mice regulate the expression of Cx43 under the action of estrogen [12]. Mice obtained by knockout Cx43 showed that after being stimulated by hyperovulation, decidualization of the endometrium was impaired and, more importantly, there was no vascular growth to maintain normal pregnancy [13]. In human embryonic stem cells, knocking out the Cx43 gene showed that some secretions controlled by Cx43 may be important factors affecting the angiogenesis of ESC [14].

Whether the communication link between cells is normal depends on two aspects: whether or not Cx is expressed, and whether the junction of the two connectors is successful (whether the gap junction channel can be opened). Cx43 is expressed in many human tissues, such as ovarian granular cells and ESC in the female reproductive system. The expression of Cx43 in the ovary and endometrium fluctuated. For example, Cx43 expression between granulosa cells was up-regulated by FSH stimulation. The LH peak

appeared after ovulation. Although Cx43 expression was unchanged, Cx43 was gradually phosphorylated, and the gap junction channel started to close. Subsequently, the expression of Cx43 was significantly decreased, and finally disappeared. Endometrial Cx43 expression in the ovulation stage was the lowest. With the endometrium into the secretory phase, the expression of Cx43 increased gradually, whereas the progesterone peaks appeared during the peak expression of progesterone [15]. In Cx43 deficient mice, osteoblasts initiated the apoptosis process, resulting in the disappearance of the response to parathyroid hormone. Therefore, it can be inferred that Cx43 can protect cells from entering the apoptosis process. Apoptosis plays an important role in the process of decidua disappearance and placental formation [16]. The expression and distribution of connexin43 in the receptive endometrium was

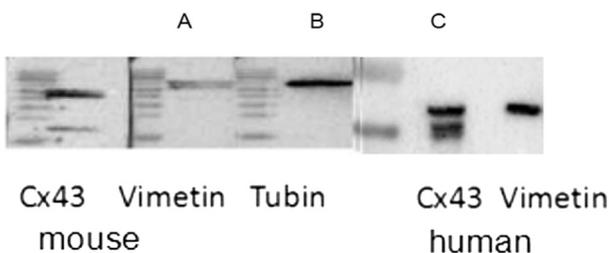


Fig. 2. Identification of mouse and human ESC: A represents mouse ESC expressing Cx43 protein and specific vimentin. B represents specific antibodies. C represents human ESC expressing Cx43 protein and specific vimentin.

gene expression in mouse

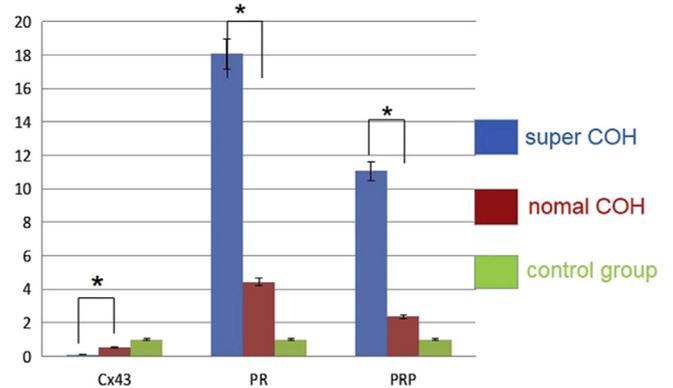


Fig. 3. Comparison of expression level of Cx43, PR, and PRP mRNA in endometrial stromal cells: normal COH means 10 IU PMSG was injected intraperitoneally in mice; super COH means 30 IU PMSG was injected intraperitoneally in mice; control COH means saline was injected intraperitoneally in mice. * means $P < 0.05$.

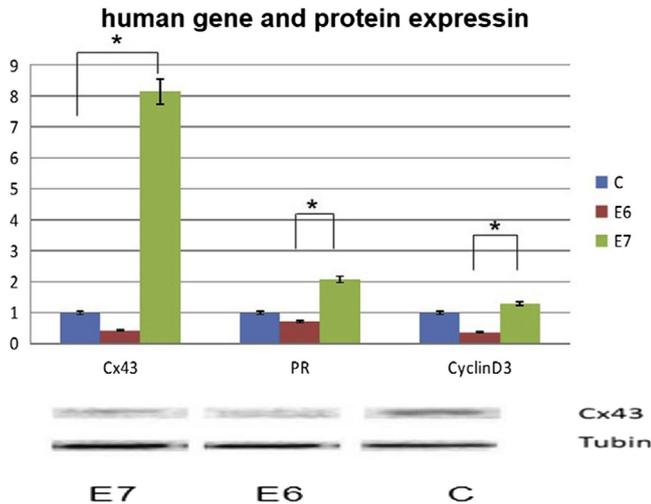


Fig. 4. Expression of genes and proteins in human ESC treated with different hormones: C means control group; E6 means the estrogen concentrations were 10⁻⁶ mol/L; E7 means the estrogen concentrations were 10⁻⁷ mol/L. * means P < 0.05.

different from that in the non-receptive endometrium. During the endometrial receptive period, intercellular communication was significantly reduced [17]. Upon appearance of the blastocyst, connecting proteins, such as Cx26, Cx43, and Cx26 began to appear locally in endometrial epithelial cells [18]. Blastocyst embryos reached the uterine cavity 4.5 days after fertilization [19], the expression of Cx26 in endometrial epithelial cells was initiated first, then the expression of Cx43 in stromal cells was induced, after which decidualization was initiated. Cx43 expression in stromal cells is inhibited by embryo implantation in the uterus [20]. The present study suggested that mouse CX43 was significantly suppressed before embryo implantation in mice with overstimulation of ovulation, which may suggest that normal expression of Cx43 plays an important role in endometrial receptivity.

Our data showed that different controlled superovulation protocols had a significant effect on Cx43 through. Our findings suggested that different concentrations of estrogen play an important role in it. Therefore, we added different concentrations of estrogen *in vitro* to simulate different controlled superovulation protocols.

Expression of the Cx43 gene and the concentration of estrogen *in vitro* were found to be dose-dependent by simulating controlled hyperovulation. The higher the estrogen concentration, the lower the expression of Cx43 gene and Cx43 protein. Thus, higher estrogen concentrations may inhibit the function of GJ.

Cx43 protein is one of the protein channels of cell GJ and plays an important regulatory role in various cellular activities [21]. Cx43 protein is one of the most important proteins in connectors. These connectors on the cell membrane gather into clusters to form GJ spots. The number of GJ plays an important role in intercellular communication. It has been suggested that there is a close relationship between endometrial receptivity and the function of intercellular junctional communication, so the function of GJ may be related to normal implantation of the embryo [22]. In a previous study, it was shown that the level of estrogen and progesterone may be closely related to the expression of Cx43 protein [23]. Expression of the Cx43 gene may increase gradually in the endometrium. During estrus, the increase in progesterone concentration may lead to the decrease of Cx43 gene expression in the endometrium. It is suggested that the expression of Cx43 may be closely related to embryo implantation. In another study, it was believed that under the regulation of luteinizing hormone, FSH and progesterone, the expression of Cx43 protein decreased

in the implantation window stage, which might be beneficial for embryo implantation [24]. Cx43 protein is the largest number of connexin in the endometrium. In a study that focused on the normal menstrual cycle of women in different periods of time, the detection of Cx43 protein in the endometrium showed that the early stage of menstrual cycle proliferation showed expression of Cx43 [25]. The expression of Cx43 protein was significantly increased in women of childbearing age with a normal menstrual cycle on the 11th to 15th day of menstrual cycle and 3–4 days before ovulation. After ovulation, the expression of Cx43 decreased, and gradually disappeared with the approach of delivery, indicating that the expression of Cx43 protein in the endometrium at different stages of menstrual cycle changes and is closely related to progesterone levels. Data by Stern et al. were similar to the findings presented in our study. In the normal menstrual cycle, the expression of Cx43 protein was weak in the early stage of proliferation and increased in the late stage of proliferation. After ovulation, the expression of Cx43 protein was inhibited at the early stage of secretion, while the expression of Cx43 protein disappeared completely at the late stage of secretion. In animal studies, expression of the Cx43 gene was affected by the estrogen (E), progesterone (P), and E/P ratio. An appropriate amount of estrogen may increase the expression of Cx43 mRNA and its protein, while progesterone may inhibit the transcription of Cx43 gene, and may inhibit the function of the golgi transport. These data also suggested that the expression of Cx43 gene may be closely related to the E/P ratio [26].

Kilarski et al. showed that the ratio of estrogen to progesterone was related to the number of interstitial connection plaques. When the ratio increased, but the number of gap junction plaques was reduced [27]. Jie et al. showed that Cx43 shRNA destroyed the gap linking channel, indicating that the decidualization of ESC did not occur [28]. The accumulation of Cx43 protein can enhance the decidualizing level under the combined action of estrogen, progesterone, and cyclic adenosine monophosphate. The estrogen concentration plays an important role in fertility, and it was found that the concentration of estrogen determines the mouse uterine receptivity period window. When the concentration range was very narrow, a long low concentration of estrogen can be maintained in the window with time, however in the high concentration of estrogen level, the window rapidly closed [29]. During embryo implantation, endometrial decidualization occurs around the ESC with blastocysts. One of the characteristics of decidualized cells is that the expression of Cx43 protein increases rapidly. The role of rapidly increasing Cx43 protein is to control the paracrine of decidualized cells and regulate angiogenesis. Decidua cannot be completed in rats with a Cx43 gene deficiency [30]. Sidell et al. argued that the growth and differentiation of ESC was facilitated by the coordinated and synchronized network of interstitial junctions between cells, particularly the gap junction protein 43, which plays an important role in rapid differentiation of ESC, the formation of blood vessels, and survival of the embryo in the implantation site of the blastocyst [31]. It was found that the mitosis of ESC was decreased, apoptosis of ESC was promoted, and the length of telomeres was shortened after interfering with a gap junction protein Cx43 channel inhibitor. The decrease of Cx43 gene expression and the obstruction of decidualization in the endometrium supported Cx43 to play an important role in embryo implantation defects and infertility. This study showed that the expression of Cx43 was related to estrogen concentrations during the process of simulating *in vitro* superovulation [32]. However, there are some limitations in this study, and endometrial receptivity is important. Moreover, decidualization of the endometrium is also important. This study did not focus on the relationship between Cx43 and decidualization of endometrium.

The low expression of connexin43 under the influence of a superdose estrogen may only be one of the factors affecting receptivity of the endometrium. Our findings indicated that the expression of connexin43 and the concentration of estrogen present a significant dose effect, suggesting that the connexin43 plays an important role in receptivity of the endometrium.

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Disclosure

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Conflict of interest

The authors had no conflict of interest.

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None.

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