Original Article

Evaluation of Progesterone and Ovulation-stimulating Drugs on the Glandular Epithelium and Angiogenesis in Mice

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Abstract

Background: Human endometrium is a dynamic tissue during the menstrual cycle can be influenced by ovarian hormones. The purpose of this study was to evaluate the endometrium angiogenesis under the influence of human menopausal gonadotropin and human chorionic gonadotropin (HMG and HCG) that stimulate ovulation and progesterone. **Materials and Methods:** In this study, thirty adult female mice were randomly divided into three groups as: control, gonadotropin and gonadotropin + progesterone. The mice in the other two groups except the control group received 7.5 IU HMG and later HCG. Subsequently, the mice were placed in a cage for mating. Gonadotropin + progesterone group was administered, 1 mg/mouse progesterone in 24, 48, and 72 h interval, after HMG injection. Ninety-six hours after HMG injection, animals were sacrificed, and their uterine specimens were prepared by immunohistochemistry technique for light microscopic studies, and statistical analysis was carried out. **Results:** Endometrium angiogenesis in control group showed that mean ± standard deviation was 24.15 ± 11.15, gonadotropin group was 62.50 ± 24.16, and gonadotropin + progesterone group was 41.85 ± 19.54. Significant difference between the control group and gonadotropin group and between the control group and gonadotropin + progesterone was observed. Statistically significant differences were observed in all groups in the endometrial angiogenesis ($P < 0.05$). **Conclusion:** Ovarian induction with gonadotropins and gonadotropins + progesterone could not change the morphometrically index of endometrial glandular epithelium in mice. Ovarian stimulation followed by progesterone injection could modify the angiogenesis of mice endometrium.

Keywords: *Angiogenesis, endometrium, implantation, progesterone*

Introduction

One of the most crucial events in reproductive period is implantation window (IW). After IW time, the embryo cannot attach to the endometrium; therefore, the most critical issue for successful implantation is timely entrance of embryo to the uterine cavity during the IW.[1] Implantation is one of the most complicated basic steps during the reproductive period that is supported by many factors. Successful implantation of embryo is a dynamic process that occurs by coordinate effects among the autocrine, paracrine, and endocrine factors.[2] Since many researches have been carried on implantation because of its importance and critical role.

Unfortunately, in assisted reproduction technology (ART) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), implantation failure is a factor that has affected the failure of these methods.[3] According to the previous studies, failure of implantation occurs from both the uterus and embryo, such as inadequate uterine receptivity is responsible to two-thirds and embryo is responsible to one-third of implantation failure.[4,5]

Endometrial thickness is an indirect marker of endometrial receptivity.[6] In recent years, endometrial thickness has much attention for researchers, and many studies were done on the effect of endometrial thickness on pregnancy rates in patients undergoing ART.[7-9]

Endometrial thickness is influenced by many factors; angiogenesis is one of the key factors involved in it. Formation and development of vessels consist of two methods: vasculogenesis and angiogenesis. Vasculogenesis refers to the initial formation of vessels from pluripotent mesenchymal cells.[10]

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Vasculogenesis mostly occurs during embryonic and fetal periods development[11] while angiogenesis is the formation of new capillary blood vessels from former vessels. Angiogenesis usually occurs during embryonic periods and also causes to fetal growth and development but in adults occurs rarely. Angiogenesis plays an important role in rheumatoid arthritis and special diseases (such as diabetes, retinopathy, and tumor growth and metastasis) and in physiological processes such as organ development, wound healing, and menstrual cycle in women. Angiogenesis is a controlled process that rarely occurs in adults except in cases such as wound healing and menstrual cycle.[12-14]

Ovarian hormones in the menstrual cycle can caused endometrial changes, so during the ovarian follicular phase, growing follicles cause to increase, and secretion of estrogen (E2) and proliferative changes occurs under the influence of this hormone in the endometrium. After ovulation, the follicle remnants, formed corpus luteum formation and secretion of progesterone, lead the endometrium into secretory phase changes.[15]

Progesterone is one of the most important hormones involved in genital system of women. The main role of progesterone is in the uterus of women. This hormone after releasing from corpus luteum causes the endometrium to prepare for implantation after ovulation and fertilization. Progesterone causes to prevent contractions of the myometrium layer of the uterus after releasing from the corpus luteum.[4]

Other roles of progesterone are increasing adjustments of calcitonin hormone in the glandular epithelium, regulation decreasing of lactoferrin protein expression in luminal epithelium, and increase the expression of MUC1 protein on luminal epithelium cells.[16]

Specialists use the human chorionic gonadotropin (HCG) to create the artificial luteinizing hormone surge for performing the IVF and ICSI procedures and cause to stimulate the oocytes reaching and ovulation.[17]

The purpose of this study was to evaluate of progesterone and ovulation-stimulating drugs on the glandular epithelium and angiogenesis in mice using markers of angiogenesis (CD31). Important aspect of this study was to evaluate endometrial angiogenesis after observation of blastocysts into the uterus.

Materials and Methods

Animals

For this study, thirty adult female Syrian mice were considered with average weight of between 25 and 30 g. To create fertilization for every two adult female mice, one adult male mouse from the same strain was selected. For each group, ten adult female mice were maintained with the same conditions in consecutive periods of 12 h light and 12 h darkness and temperatures of 23°C ± 1°C in the animal's house of Center of Physiology, School of Medicine, Isfahan University of Medical Sciences. Then, all of the adult female mice were designed in three groups, randomly (control = Group 1, gonadotropin = Group 2, and gonadotropin + progesterone = Group 3) using simple random sampling. Urban water and Pars food were used to feed the mice.

Drugs

In this study, we used HMG 7.5 IU intraperitoneal (IP) and HCG 7.5 IU IP for superovulation of mice in two experimental groups. In one experimental group, we used progesterone (1 mg/mouse IP). According to the previous study and regard to the effects of this drug, we used progesterone for evaluating its role in endometrial angiogenesis.

Periodic acid–Schiff staining

In this method, after the Schiff reagent preparation and tissue sections (5 micron) washing in running water, samples were placed in solution of periodic acid. Then, tissue sections once more were washed in running water. Harris hematoxylin was used to identify the nuclei of cells. Finally, tissue processing steps including dehydration, transparency, and mounting were done. After ending the preparation, the periodic acid–Schiff (PAS+) tissue samples and cell cores were observed (microscopic) reddish purple and blue, respectively.

Morphological and morphometrical methods

In this study, to measure morphologically, we estimated the height of luminal cells by software Motic Image Plus 2.0 (Motic Group Co., LTD, Hong Kong, China). For morphologic examination, tissue samples were prepared and analyzed by light microscope with X660 magnification.

Immunohistochemical staining

Immunohistochemistry (IHC) method was used to determine the numbers of endothelial cells and the amount of angiogenesis, and sections were cut with 3 μm thickness. In this method, after deparaffinization, the tissue sections were incubated by the primary mouse monoclonal antibody CD31 from the Abcam Company.

According to the standard methods, the samples were prepared by the following steps:

- Deparaffinization
- Hydrogen peroxide using
- Protein block using
- Primary antibody using
- Horseradish peroxidase polymer using
- Secondary antibody using
- Using the diaminobenzidine for reveal the distribution of antibody
- Using the Harris hematoxylin for increase the background contrast
- Pasting and observing the samples in the end of the tissue sections of endometrium were examined under the Olympus light microscope with ×40 magnification

- Endothelial cells stained with CD31 randomly for each tissue sample in selected fields were counted. In this method, endothelial cells are observed as dark brown using this marker.

Statistical analysis

Capillary density expressed as CD31-positive cells/filed ×40 in the endometrium of animals. After counting of cells CD31+, numbers endothelial cells and height of glandular epithelium (from basal lamina to apical surface) (Motic Image Plus 2.0. software) were statistically analyzed by one-way ANOVA and least significant difference (LSD) (all groups were compared with each other). The data were statistically analyzed using SPSS Inc., Chicago, IL, USA 16. $P < 0.05$ was considered statistically significant.

Results

According to our study, in the control group, luminal epithelium was observing to show a long cylinder and contained numerous PAS+ granules that the granules were scattered on the entire surface of epithelium but was observed more in the basal region of the cell.

In the gonadotropin group and gonadotropin + progesterone group, endometrial thickness shows slight increase compared to the control group. The epithelium shape and the amount of PAS+ granules were similar to the epithelium in other groups. In all groups, the endometrial glandular epithelium in the luteal phase and cell nuclei located in the central area.

The mean height of glandular epithelium cells was 11.66 ± 1.54 μm in control group, 11.10 ± 0.77 μm in gonadotropin group, 11.06 ± 1.30 μm in gonadotropin + progesterone group. Finally, the heights of the cells in all groups were not significantly different from each other ($P < 0.05$ for all of the comparisons) [Figure 1].

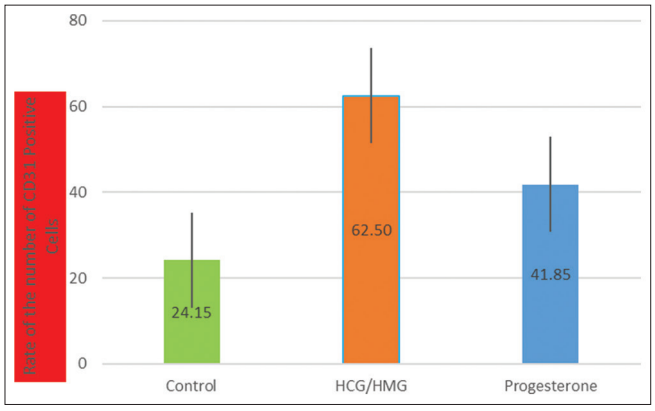

Figure 1: Graphical comparison of the mean height of glandular epithelium cells between the three groups. As is indicated, there is a significant difference between the three groups. Furthermore, significant difference is observed between control and gonadotropin, control and gonadotropin + progesterone, and gonadotropin and gonadotropin + progesterone (criteria: Micrometer, $P < 0.05$)

Using the IHC method, the number of endothelial cells that was counted using CD31 marker using the Olympus light microscope with ×40 magnification showed a significant difference among the three groups. In this method, using the CD31 marker of endothelial cells appears to dark brown color.

According to the results of counting the number of endothelial cells in randomly selected fields, the average of the rates was 24.15 ± 11.15, 62.50 ± 24.16, and 41.85 ± 19.54, respectively, in control, gonadotropin, and gonadotropin + progesterone groups.

Significant differences were observed between the three groups using the statistical software of SPSS, Chicago, IL, USA 16 and by one-way ANOVA and LSD. $P \leq 0.05$ (in this study our $P \leq 0.01$) indicates a significant difference and showed a significant difference between mean ± standard deviation.

We each group compared with the other groups. Significant difference was observed between the control group and the gonadotropin group. Significant difference was observed between the control group and the gonadotropin + progesterone group and also between gonadotropin + progesterone and gonadotropin group ($P \leq 0.05$).

According to the above description and Figure 2, increase rate of the number of endothelial cells in the ovulation stimulated group is more than double compared with the control group. This rate was higher compared with gonadotropin + progesterone as well as the differences are depicted in Figure 2.

However, in this [Figure 2], the number of endothelial cells in the progesterone group had a significant increase compare with control group, but it was much less evident than the gonadotropin group. With respect to Figure 3, the number of endothelial cells was observed in the control group. As is clear in the figure, the rate of endothelial cells and capillary density in this group is observed low.

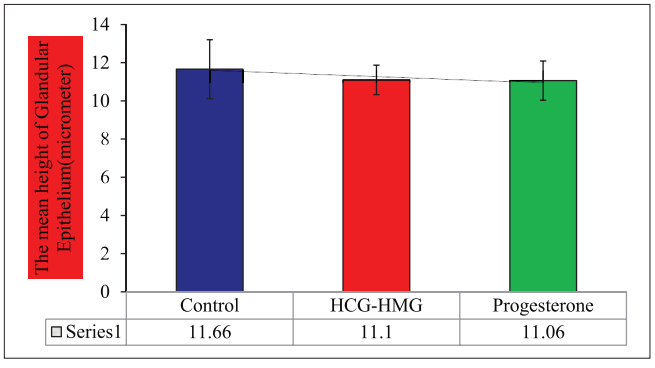

Figure 2: Graphical comparison of rate of the number of CD31-positive cells between the three groups. As is indicated, there is a significant difference between the three groups. Furthermore, significant difference is observed between control and gonadotropin groups, control and gonadotropin + progesterone groups, and gonadotropin and gonadotropin + progesterone groups (number of CD31-positive cells, $P < 0.05$)

In Figure 4 that the rate of capillary density is extremely high, it is related to gonadotropin group. The use of drugs that stimulate ovulation can increase the number of endothelial cells and angiogenesis. In Figure 5 that is related to the gonadotropin + progesterone group, the capillary density has also increased compare to the control group. Endothelial cells were observing to increase in Group 3 showing process of angiogenesis quality as positive [Figure 5].

Discussion

The results revealed that angiogenesis parameter can be changed affected by exogenous factors such as gonadotropins and progesterone. The use of gonadotropins in the ovulation-stimulating process increased angiogenesis in this group in compared with control group. In the other group, the use of gonadotropins + progesterone caused to reduce the angiogenesis compared with gonadotropins group. According to these results, the inhibitory role of progesterone on angiogenesis can be inferred.

However, gonadotropins also stimulate the angiogenesis. Macrow *et al*. in a study show the effect of superovulation agonists using a gonadotropin-releasing hormone (GnRH-a) and HMG and the structure of endometrial HCG. There was no difference in the development of endometrial glandular by both criteria standard morphometric analysis or evaluation.[18] Macrow PJ *et al*. studied the size and thickness of the endometrial glands in patients receiving HCG, HMG, and GnRHa in the early luteal phase and did not see the changes in these parameters.[19] These results match with our results.

Angiogenesis in endometrium of the uterus is a process affected by many factors. In general, vascular proliferation occurs in the endometrial surface area of human and other mammals.[20] Blood vessel growth in the endometrium is consisted of complicate mechanisms. According to the other comments, researchers were compared three mechanisms of angiogenesis, including sprouting, intussusception, and elongation.[21,22]

The researchers also stated that elongation of vascular is the main mechanism of endometrial angiogenesis of the uterus during early to mid-secretory phase.[21] In the mid to late proliferative phase and early to mid-secretory phase of the cycle, the length density of new vessels has the highest amount of itself, also the new blood vessels based on length-volume ratio during the early proliferative phase and the mid to late proliferative phase growth more quickly than its surrounding tissue.

These patterns were similar in each region of the endometrium, and the results showed that estrogen in the proliferative phase is cause of further vascular elongation while vascular remodeling occurs during the secretory phase. Other researchers also presented the evidence on vessel elongation during the early proliferative phase.[23]

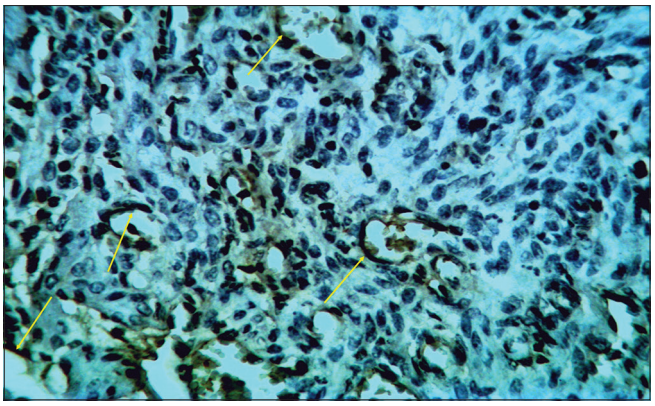

Figure 3: The number of endothelial cells in the group control, immunohistochemical staining of CD31-positive cells. Magnification × 40/endothelial cells are shown with arrows

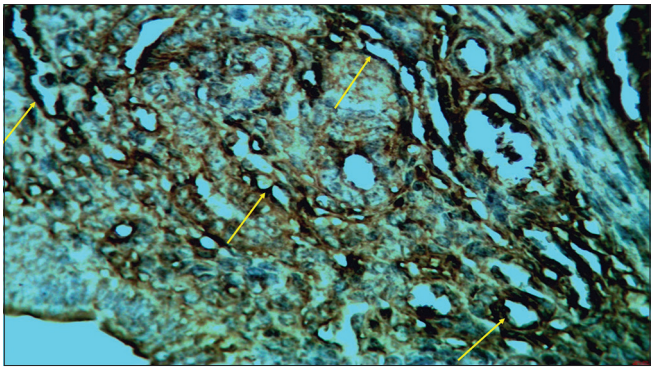

Figure 4: The number of endothelial cells in gonadotropin group, immunohistochemical of CD31-positive cells. Magnification × 40/endothelial cells are shown with arrows

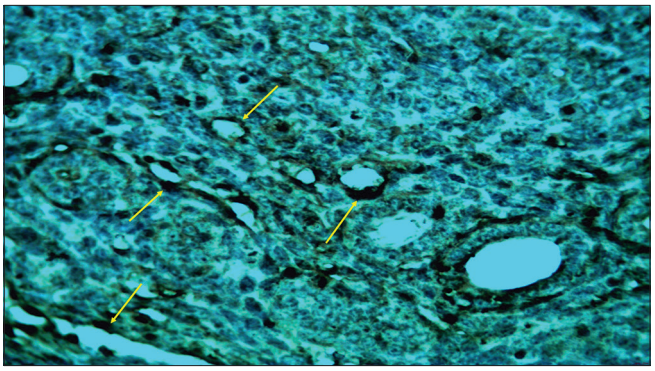

Figure 5: The number of endothelial cells in gonadotropin + progesterone group, immunohistochemical of CD31-positive cells. Magnification × 40/endothelial cells are shown with arrows

With using the rhesus monkey model has been shown that the angiogenesis has the highest dependency to the estrogen, while the proliferation of endothelial cells occurs in the menstrual phase, also exclusion and removal of estrogen and progesterone cause to decrease in endothelial cell proliferation.[21]

Density of blood vessels due to growth of the endometrium parallel to angiogenesis during the cycle is an important factor for determination of the extent of the cycle and

the process of angiogenesis. Although this measure in humans and baboon monkeys is not able to determine the cycle progression, its reason is that the results may be affected by changes in the volume of edema and cellular components.[24,25]

In a study that was done on endometrial glandular epithelium, results showed that morphometric (height of luminal epithelial cells) endometrial glandular epithelial cells did not have any change in the ovulation stimulated and gonadotropin + progesterone.[26]

However, in another study, ovulation stimulating with progesterone did not improve perfection of pinopodes.[27]

The results of another study we are looking for the effects of progesterone on endometrial epithelial cell morphology and after ovulation induction to improve maturation of the endometrium.[28]

In similar studies, with due attention to the critical role of angiogenesis process, researchers studied the effect of the drugs on the endometrium to prevent the implantation failure in ART treatment with creation the proper thickness. Retinoic acid is one of these drugs that in endometrial stromal cells cause to enhance the secretion of vascular endothelial growth factor (VEGF) by a mechanism of hypoxia activation through a combination of transcriptional activators of VEGF. These results show that there is a strong correlation in the spatial variations between synthetic retinoid and stromal in the preimplantation stage and cause to regulate the VEGF secretion and induce early angiogenic events during pregnancy.[29]

In other studies, using dienogest causes to inhibition of angiogenesis and makes structural changes with it in the microvilli. According to our studies, diabetes reduces the angiogenesis and prescription of GW0742 as PPARβ/δ agonist leads to improvement in the angiogenesis. Although our study was on the heart muscle, it is possible that this be also true in the case of uterus endometrial.[30,31]

According to the direct role of the HCG hormone in the regulation of the implantation and development of placenta, this hormone stimulates VEGF expression and therefore has an indirect role in the angiogenesis rising.[32,33] Measurement of urinary VEGF concentrations in women under fertilization in the *in vitro* environment showed increasing of VEGF concentrations. This increase in VEGF concentration of serum and follicular fluid was also improved.[34,35]

Estradiol and progesterone cause to increase the expression of Ang-1 by glandular epithelium and also proliferation of smooth muscle cells of small vessels in the endometrium of the rhesus monkey[36] and caused to maturation of vascular by increasing wall coverings in uterus endometrial of mouse. Effect of ovarian hormones on the process of angiogenesis during different stages of the menstrual cycle has a different pattern.[37] Vessel sprouting is stimulated during the secretory phase and in the presence of progesterone.[38] In surveys conducted in the *in vitro* environment, progesterone was identified as an inhibitor of endothelial cell proliferation. This hormone causes to stop the cell cycle during the G0/G1 phase of dermal endothelial cells. Human endometrial endothelial cells) HEEC) display increased proliferative and angiogenic activities in response to ovarian steroids. This results suggest direct effects of E2 and P4 on HEEC function, providing additional understanding of physiological role of the endothelium in endometrial function.[39] Progesterone had less effect on vessels permeability and cause to maturation and differentiation of the vessels.

Finally, we can say that access to a suitable angiogenesis in endometrium of the uterus, cause to increase improve the performance of implantation, and create proper thickness of the endometrium for this crucial function.

Conclusion

According to the results of this study can be concluded that use of the gonadotropins stimulates the endothelial cells proliferation and increases the capillary density. Ovarian induction followed by gonadotropin and progesterone injection would not modify the morphometrical indices of glandular epithelium of mouse endometrium. However, results of the gonadotropin + progesterone group determined the inhibitory role of progesterone on endothelial cells proliferation. Undoubtedly, the use of endothelial cells proliferation-enhancing drugs will be able to create an effective angiogenesis in the endometrium. Achieving to a proper angiogenesis will improve the receptivity of endometrium and will prevent implantation failure.

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Conflicts of interest

There are no conflicts of interest.

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