Research Paper: GONAL-F Induced Endometrial Thickness Changes in Ovariectomized Rats With and Without HCG

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ABSTRACT

Introduction: To attach the fetus to the endometrial epithelium, the endometrium should be affected and allowed by the steroid hormones secreted from the ovary. Thus, the endometrium is then subject to several structural and biochemical changes. Implantation involves the embedment, adhesion and invasion steps that specific receptors are engaged in that. The role of membrane receptors in uterine epithelium is important. Most of these receptors are glycoprotein and among which integrins are of importance. In menstrual period, the uterus undergoes structural changes, so that endometrium is provided for the placenta and the embryo in addition to extracellular matrix, especially fibronectin.

Methods: The animals were anesthetized with ketamine and xylacin intraperitoneally, to perform surgery. After opening the abdomen, the ovaries were removed from both sides. After 72 hours, and observing the vaginal plaque, animals received high doses of ketamine and xylazine. The left uterine horn was removed and placed in fixative solutions and prepared for cutting during the tissue processing of the molds. The slices were stained with PAS to determine the glycoprotein layer thickness.

Results: There is a statistically significant difference in the increased luminal membrane thickness of glycoprotein layer of endometrial epithelium cells between the Gonal-F receptor groups with hCG, compared to other groups at the same time.

Conclusion: The study results indicated that Gonal-F and hCG can increase the amount of glycoprotein expression without damaging the endometrial wall.

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1. Introduction

In the erythrocyte secretion phase, ovulation occurs on the 14th day in a typical 28-day cycle. With the onset of progesterone secretion in 48-72 hours after ovulation, the appearance of endometrial histology changes to secretion state. The naming of this stage is due to the presence of eosinophilic and protein-rich secreted material in the gland ducts. The secretory phase of the menstrual cycle is characterized by the effects of estrogen and progesterone. Generally, progesterone has anti-estrogen effects. In this phase, estrogen receptors significantly decrease in endometrial cells.

During the secretory phase, the endometrial glands form their own glycogen vacuoles. These vacuoles are PAS- positive staining. They initially appear in a subcortical position, then move to the gland ducts. On the 17th day of the cycle, vacuoles can be placed in the middle of cells. They eventually enter the gland ducts as euphorbine secretion in the 20th day of the cycle. On the sixth to seventh days after ovulation, the secretion of the glands reaches its maximum, and endometrium receives the optial condition for blastocyst implantation.

In order to receive the embryo, the endometrium has many structural and biochemical changes that are related to hormones and gene expression regulation. Morphological changes include changes in the plasma membrane [1]. Accordingly, the shape and appearance of apical membrane microvilli, the number and depth of connections between epithelial cells in the lateral membrane are changed. Also, the base membrane changes appear in the form of an increase in the base laminate thickness and membrane and cellular skeleton folding [2], which ultimately lead to the possibility of implantation [3].

At the secretory phase , the stroma remains unchanged until the seventh day after ovulation, in which develops progressive edema. In the secretory phase along with maximum stromal edema, the spiral arteries are observed clearly. These arteries become longer and more spiral during the remainder time of secretory phase. Almost 2 days before menstrual bleeding, the number of multi-core lymphocytes that migrated from vascular system increase significantly. This increase in leukocytes indicates decreased endometrial stroma and the onset of menstrual period [4].

Implantation involves blastocyst embedment on endometrial surface epithelial cells in secretory phase and its proteolytic penetration into the stroma, which lasts about 3 days. This action depends on the adhesion, embedment and penetration mechanisms that multiple molecules and receptors play roles in uterine epithelium and embryonic cells in this process. Surface L-selectin in trophoblast cells and relevant carbohydrate receptors of uterine epithelium are the primary link between the blastocyst and uterus [5, 6].

More trophoblast linkage and penetration into the uterine tissue is met by the membrane receptor of the uterine epithelium [7]. Most receptors are glycoproteins, but the integrins include cellular connector, which is of particular importance among them. Implantation depends on the relationship between trophoblast adhesion and extracellular matrix [3, 8]. Various integrin and fibronectin are expressed in endometrium to a large extent, and engage in the implantation process [9].

Fibronectin is a large filamentous glycoprotein strain, mainly produced by the fibroblasts of the heterodimer. Fibronectin and integrin are effective in cell migration and intercellular adhesion [10]. This glycoprotein is soluble in the plasma, also comes in the form of a strand in the extracellular matrix, which is linked to the integrin to perform the discussed activities [11]. Integrins are heteromodromic glycoproteins, composed of α and β subunits with a large family of 24 known members that allow mammalian cells for tracing the migration pathway, location, differentiation, growth, and polarity. They also act as a connection harbor for cells [12].

Integrins undergo structural changes throughout the various phases of menstrual cycle and, prepare the endometrium for the placenta and embryo by adding extracellular matrix materials, especially fibronectin [5-6]. β1 integrin can link to different subgroups of α, and heterodimers like β1 α5 [13] and cause β1 α3 act as a receptor of fibronectin [14]. The transmission pathways between integrin, fibronectin, and intracellular proteins such as thalene, vincoulin, and actin that are involved in cell adhesion are identified [12].

2. Materials and Methods

The reproductive cycle of rats consists of 4 stages of proestrus, estrus, metestrus and diestrus. The onset of puberty in the female rats occurs during the fourth week of life, in which LH secretion occurs that causes the ovum to mature. These changes occur in the secretion of LH about 8-9 days before the first prosthesis. Then, the first stage of proestrus occurs. During the whole year, the rat's ovulation occurs every 4-5 days. In a 4- to 5-day cycle, the rat has 14 to 12 hours of proestrus, 25-27

hours of estrus, 6-8 hours of metestrus and 55-57 hours of diestrus. In other words, each period has its own day. However, the epithelium layer that matters here experiences a lot of changes at the various stages of estrus [15]. Therefore, the adult female mice were used and anesthetized by ketamine (100 mg/kg) and xylalin (10 mg/kg). Left uterine horn was removed through surgery. Animals were randomly divided into 3 experimental (n=35), sham (n=5) and control (n=5) groups.

The experimental group was divided into 7 subgroups based on the dosage of Gonal-F (IU) with and without hCG, and hCG alone, titled as follows: 1. Gonal-F 10 recipient group (G10); 2. Gonal-F 20 recipient group (G20); 3. Gonal-F 30 recipient group (G30); 4. hCG recipient group; 5. Gonal-F 10 recipient group with hCG (H+G10); 6. Gonal-F 20 recipient group with hCG (H+G20); and 7. Gonal-F 30 recipient group with hCG (H+G30).

Gonal-F dosage was intraperitoneally injected to the experimental group for 5 days and after receiving the last Gonal-F dosage, the hCG group were injected with the same method. After 72 hours and observing the vaginal plaque, the animals were anesthetized using a high dosage of ketamine (150 mg/kg) and xylacin (15 mg/kg) by intraperitoneal injection. Samples were separated and the tissues were placed in 10% formalin solution. In the tissue processing stage, the tissues were prepared for molding in dehydration, clarification, hydration and impregnation.

The molds were then prepared for cutting with a microtome and transduction was executed on the slide. Slides were prepared for acid-Schiff staining, after deparaffinization. In this staining protocol, the slides were first in xylene and then hydrated in absolute alcohols up to 70%. Then, samples were washed with distilled water. In the following step, the slides were placed in a periodic acid solution for 5 minutes and then rinsed with running water. Then, the slides were placed in a dark environment for 15 minutes and rinsed with gentle running water for 15 minutes until the specimens became completely red. After this step, the slides were placed in a hematoxylin acid solution for 1 minute for differential staining and rinsed with running water. Eventually, the slides were placed in xylene jar again. The slides were mounted and sealed with Antalan glue.

In order to study the tissue changes in the studied groups, the cuttings with 3 to 5 paraffin blocks and at least 8 sections from each block were examined in each group. Four images with 400× magnification were taken from each cut and the cut surface was divided into 4 equal squares. The numbers of stained cells with the reagent was checked by the optical microscope in each square and the thickness of the glycoprotein layer of the cell was measured. The calculations were performed by ImageJ software and the results were announced after examining the slides.

3. Results

The thickness of the glycoprotein layer of the luminal membrane of the endometrial epithelium cells, Mean±SD variation of the glycoprotein layer thickness of luminal membrane of endometrial epithelium cells in all of the studied groups are listed in Table 1 and Figure 1 (The highest thickness is observed in the groups of G20+hCG, hCG).

We compared Mean±SD of the glycoprotein layer thickness of luminal membrane of uterine endometrial epithelium cells in micrometers among all the studied groups. Results indicate a statistically significant difference in the thickness of the glycoprotein layer of luminal membrane of uterine endometrial epithelium cells in micrometers, among all the experimental groups and the control, sham and hCG groups ($P<0.00$). There is a statistically significant difference in the glycoprotein layer thickness of luminal membrane of uterine endometrial epithelium cells among Gonal-F recipient groups and the control, sham and hCG groups ($P<0.00$).

According to Tukey test, there is a statistically significant difference between the study groups except for the sham and control groups. The glycoprotein layer thickness of luminal membrane of uterine endometrial epithelium cells among the recipient groups (G10, G20, G30) decreased significantly, compared to the control, sham and hCG groups ($P\leq0.0.00$). The glycoprotein layer thickness of luminal membrane of uterine endometrial epithelium cells decreased significantly among the recipient groups (G10, G20), compared to the control, sham and hCG groups, and increased significantly compared to the G30 group ($P\leq0.05$).

There is a statistically significant difference in the glycoprotein layer thickness of uterine endometrium in Gonal-F recipient without hCG, compared to the Gonal-F recipient groups with hCG ($P<000$). The glycoprotein layer thickness in Gonal-F recipient groups with hCG (G10+hCG), (G20+hCG) and (G30+hCG) showed a significant decrease ($P\leq0.000$), in comparison with the control, hCG and sham groups. As shown in Figure 2, there is a significant difference in the glycoprotein layer thickness among all the test groups and the control group, except for sham and hCG groups. The glycoprotein layer thickness is determined by an arrow in the Figure 2.

Table 1. The mean of the thickness of the glycoprotein layer

	Control	Sham	G10	G20	G30	G10.hCG	G20.hCG	G30.hCG	hCG
Mean	5.0992	5.1450	2.6542	2.3675	1.8617	3.4317	3.4229	3.1696	4.0546
SD	0.42520	0.30835	0.32194	0.35078	0.29563	0.38567	0.46732	0.43454	0.31378

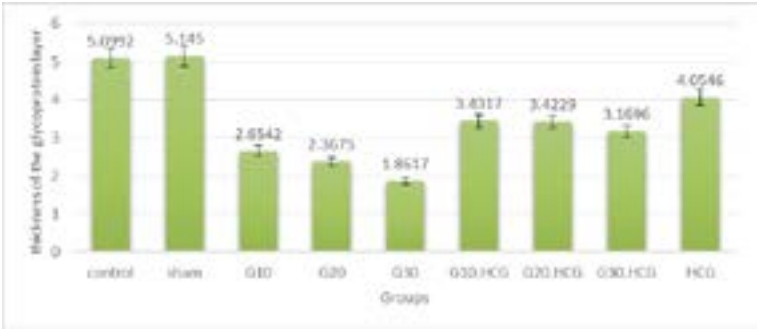

Figure 1. The mean of the thickness of the glycoprotein layer

Significant differences are observed in the glycoprotein layer thickness of luminal membrane of uterine endometrial epithelium cells among the Gonal-F recipient groups, compared to the control, sham and hCG groups ($P<0.05$). There is no significant differences between the sham and control groups ($P=0.997$*). Thickness in the recipient groups (G10, G20, G30) is significantly lower than the control, sham and hCG groups ($P\leq0.0.00$). Thickness in the recipient groups (G10, G20) is significantly lower, compared to the control, sham and hCG groups, and is significantly higher compared to the G30 group ($P\leq0.05$).

The obtained data showed a significant decrease in the groups receiving Gonal-f with hCG (G10+hCG) (G20+hCG), (G30+hCG) compared to the control, hCG and sham groups ($P\leq0.000$). There is no statistically significant difference between the 3 recipient groups (G10+H), (G20+H) (G30+H). There is a statistically significant difference between the hCG recipient groups, compared to the control and sham groups ($P<0.001$). There is a significant difference in the glycoprotein layer thickness of uterine endometrium in the groups receiving the Gonal-F 10,20,30 and the groups receiving Gonal-F plus hCG.

The glycoprotein layer thickness of luminal membrane of uterine endometrial epithelium cells of the Gonal-F receptor group with hCG is greater than the same in Gonal-F 10-,20-,30 recipient groups ($P\leq0.000$). The recipient groups (G10, G20, G30) showed the highest decrease in thickness, compared to the control and sham groups. The One-way ANOVA and Tukey tests were used to compare the groups at a significance level of $P\leq0.05$ (Table 2 and Figure 3).

4. Discussion

Implantation is a complex process with various steps in which the cell-cell connection between trophoblast cells and endometrial epithelial cells is of particular importance. Obviously, endometrium is affected by estrogen and progesterone. Disruption of these hormones affects endometrial morphology [16], and endometrial histology and disturbs endometrial receptivity. It has also a negative impact on the integrin [17]. Integrin plays a vital role in implantation. The α1 and β3 integrins are reduced in the infertile people [18].

Fatih et al. studied the effect of Gonal-F with doses of 10 and 20 units on the expression of α1 and β3 integrins and noted the decreased expression of integrins [19]. Considering that the integrins are part of glycoproteins, a significant reduction in glycoproteins thickness in groups receiving the Gonal-F compared to the control, sham and hCG groups is due to the effect of the drug on the expression of integrins. By increasing the Gonal-F dosage, glycoprotein levels reduced and its optimal dosage is observed in low dose which was not discussed in the prior research. Furthermore, adding hCG to the Gonal-F treatment protocol at 10, 20 and 30 dosages,

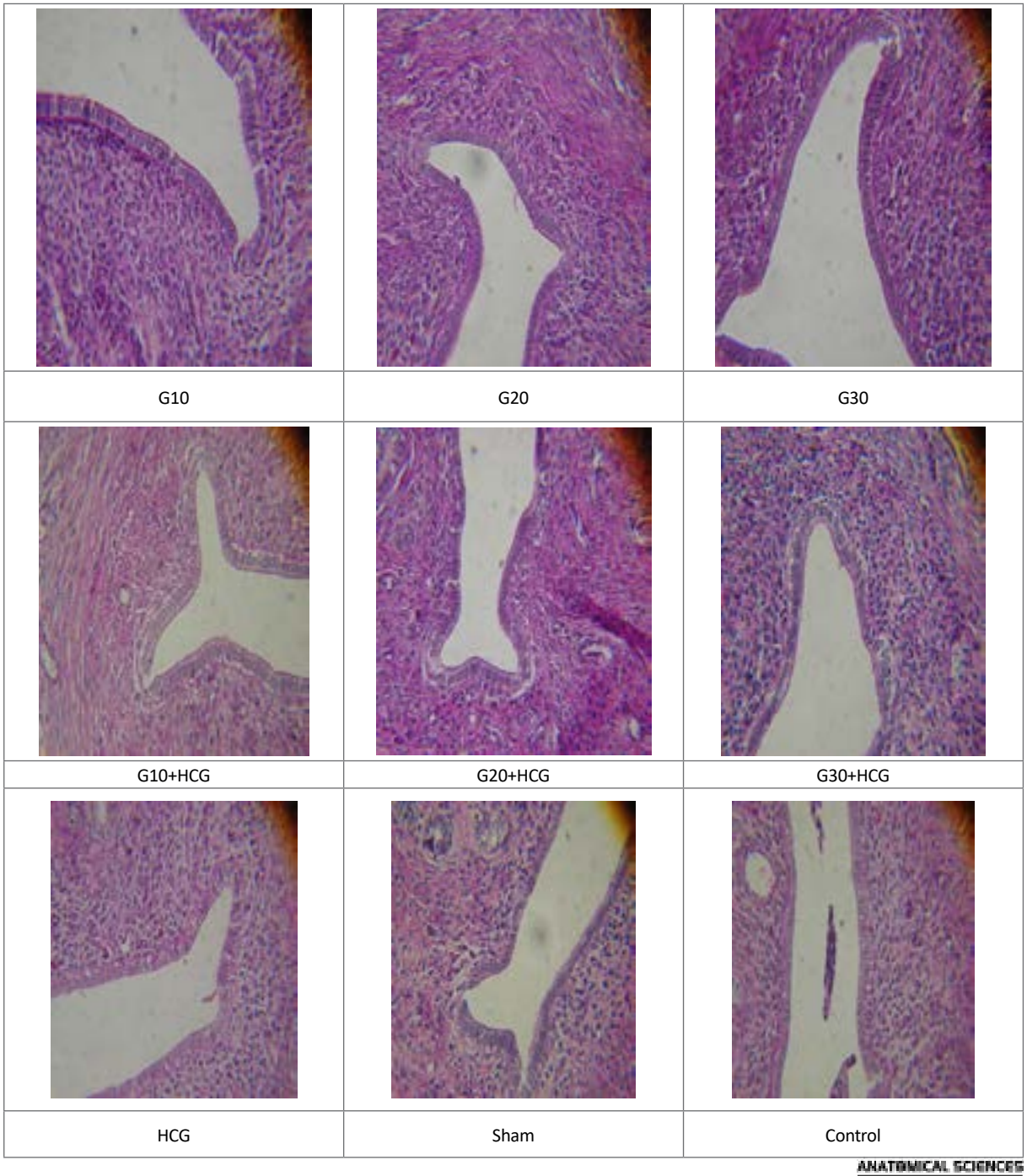

Figure 2. Staining glycoprotein of endometrial epithelium cells with periodic acid Schiff with a magnification of 400

resulted in a significant increase in glycoprotein receptor intake, compared to the Gonal-F treatment protocol without hCG.

The highest increase is observed at dosage of 10, even though being low, compared to the natural groups. Qiuju who confirms the study results of Chen et al. provided a remedy for Gonal-F and hCG to patients undergoing IVF and noticed a decrease in the expression of β3 and LIF integrins in the implantation window [20]. The result obtained in this study is that in groups receiving the GFR and hCG showed a significant difference in the reduction of glycoprotein expression with the control and hCG groups which can be the result of the effect of the drug on glycoproteins such as integrins. The glycoprotein glycodillin is a superficial glycoprotein in the reproductive system tissues of the human, baboons and prostate [21] and has different isoforms.

Table 2. The Mean±SD changes in the size of the thickness of the glycoprotein layer of the uterine endometrium

Group Mean± SD	Control 5.0992± 0.42520	Sham 5.1450± 0.30835	G10 2.6542± 0.32194	G20 2.3675± 0.35078	G30 1.8617± 0.29563	G10. hCG 3.4317± 0.38567	G20. hCG 3.4229± 0.46732	G30. hCG 3.1696± 0.43454	hCG 4.0546± 0.31378
Control 5.0992± 0.42520	—	P=0.997*	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000
Sham 5.1450± 0.30835	P=0.997*	—	P<0.000	P<0.000	P<0.000	P=0.011	P<0.000	P<0.000	P<0.000
G10 2.6542± 0.32194	P<0.000	P<0.000	—	P<0.045	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000
G20 2.3675± 0.35078	P<0.000	P<0.000	P<0.045	—	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000
G30 1.8617± 0.29563	P<0.000	P<0.000	P<0.000	P<0.000	—	P<0.000	P<0.000	P<0.000	P<0.000
G10. hCG 3.4317± 0.38567	P<0.000	P=0.011	P<0.000	P<0.000	P<0.000	—	P=1*	P=0.199*	P<0.000
G20. hCG 3.4229± 0.46732	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	P=1*	—	*P=0.231	P<0.000
G30. hCG 3.1696± 0.43454	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	P=0.199*	P=0.231*	—	P<0.000
hCG 4.0546± 0.31378	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	P<0.000	—

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Glycoprotein glycodillin type A (Gd-A) is found in decidua endometrium of amniotic fluid and maternal serum [22]. This type of glycoprotein plays an important role in the embryonic adhesion and inhibition of the immune system of the mother against the embryo [23]. A study reported that the adjunct hCG to the culture medium of decidua and embryonic cells caused an increase in the surface glycoprotein expression and glycoline [24]. Consistent with these studies, the present study indicated that the amount of apical glycoproteins of the endometrial cells increased significantly in the hCG consumer groups, compared to the other groups, except for the

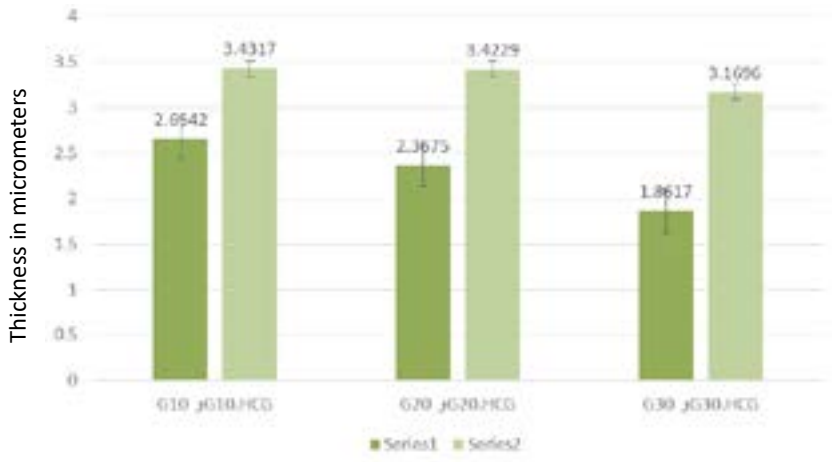

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Figure 3. The Mean±SD changes in glycoprotein layer of the uterine endometrium

control and sham groups. This could be explained by the effects of hCG on the extracellular matrix of epithelium endometrium which increases glycoproteins, important in preparing endometrium for implantation.

The secretion regulation of materials like MMP2 (Matrix Metaloproteinase2) by the hCG hormone that affects extracellular matrix components formation and increases the glycoprotein components, effective in cellular reception in extracellular matrix is documented by Tapia et al. [25]. In groups receiving Gonal-F increasing the dosage from 10 to 20 with the adjunct hCG, increased the glycoprotein thickness. However, glycoprotein thickness was reduced by increasing the dosage in groups receiving Gonal-F alone, which needs further investigation.

Given that the results obtained in this study are consistent with similar research studies, further research on higher dosage in suggested for future investigations. In addition, the positive or negative effects on increasing glycoproteins thickness caused by increasing the dosage should be addressed. In addition, the commonly used treatments should also be considered and reviewed, and their effects should be investigated, separately.

Considering that ovulation stimulants are very diverse and it is not possible to study all of them in the current study, the effects of these drugs, such as Follitropin Beta and Letrozole, which are currently in use, on the quality of endometrial factors affecting Implantation can be a useful topic for future research. Because Gonal-F has been shown to change in endometrial morphology, the effect of this drug on the morphology of epithelial cells, which is one of the effective factors on implantation, can be studied. On the other hand, the effect of hCG hormone in higher doses varies with the factors that can be induced in other studies.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. The participants were informed about the purpose of the research and its implementation stages; They were also assured about the confidentiality of their information; Moreover, They were allowed to leave the study whenever they wish, and if desired, the results of the research would be available to them.

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Authors contributions

All authors have read and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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