Original Article

Endometrial Expression of Angiogenesis-related Factors Following Ovarian Stimulation and Epigallocatechin Gallate Administration

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Abstract

Background: AngiomiRs are a specialized subclass of microRNAs that target genes related to angiogenesis, such as Vascular Endothelial Growth Factor (VEGF). Angiogenesis is crucial in all stages of pregnancy and is essential for creating a receptive endometrium for embryo implantation. Both ovarian stimulation and Epigallocatechin Gallate (EGCG), a major component of green tea, can influence angiogenesis. This study aims to examine endometrial quality, with a focus on angiogenesis, as well as serum levels of estradiol and progesterone, following EGCG administration in ovarian-stimulated mice, as a valuable model for studying human reproductive health and diseases. **Methods and Materials:** Forty adult female mice were assigned to four distinct groups: 1) control, 2) ovarian stimulation (7.5 IU HMG followed by 7.5 IU HCG 48 hours later, administered intraperitoneally), 3) EGCG (5 mg/kg EGCG for 4 days, IP), and 4) ovarian stimulation + EGCG groups. Gene expression analysis of miR-16-5p was performed using real-time polymerase chain reaction. VEGF protein and CD31-positive cell density were assessed through immunohistochemistry, and serum estradiol and progesterone levels were measured using ELISA. **Results:** Endothelial cell density, VEGF protein, and miR-16-5p expression and estradiol concentration significantly increased in the ovarian stimulation group compared to the control group ($p < 0.05$). The smallest reduction in these parameters was observed in the group that received EGCG. EGCG also significantly reduced the progesterone level ($p < 0.05$). **Conclusion:** EGCG significantly reduced endometrial angiogenesis, and angiomiR-16-5p may mediate the effects of EGCG on endometrial quality; however, further studies are needed.

Keywords: *Endometrium, epigallocatechin gallate, MicroRNAs, ovary, vascular endothelial growth factors*

Introduction

Endometrial receptivity during the implantation window requires sufficient angiogenesis.[1] MicroRNAs (miRNAs) are classified as small noncoding RNAs, typically ranging from 19 to 25 nucleotides in length.[2] The term 'angiomiR' is adopted to name miRNAs that regulate genes involved in angiogenesis-related pathways, the formation of new blood vessels from pre-existing ones. Thus, they may be novel therapeutic agents targeting angiogenesis-related conditions.[3] So, angiomiRs may stand out as potentially vital determinants in female fertility.[1]

Vascular Endothelial Growth Factor (VEGF) is a key cytokine in vascular biology, playing a central role in regulating angiogenesis. VEGF exists in several isoforms with distinct biological activities, acting through its receptors to initiate intracellular signaling cascades, including phosphoinositide 3-kinase (PI3K), p38/mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK)/paxillin, PLCγ, and Ca^{2+} signaling.[4] The VEGF signaling network induces endothelial cell proliferation, migration, and vascular permeability, while also impacting immune cell recruitment and tissue repair processes. Dysregulated VEGF signaling is implicated in various pathologies, and anti-VEGF therapies have emerged as promising interventions for cancer and ocular diseases, highlighting its clinical significance.[5,6] MiR-16-5p located on human chromosome 13q14 is known as an angio-miRNA targeting VEGF mRNA, modulating endometrial angiogenesis, and is expressed in human and mouse endometrium.[7,8] Dysregulation of miR-16-5p has been reported in pathological

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conditions such as endometriosis, preeclampsia, and endometrial cancer.[9]

In Assisted Reproductive Technology (ART), stimulated cycles are induced to reach a suitable number of mature follicles. Human Menopausal Gonadotropin (HMG) and Human Chorionic Gonadotropin (HCG) are used in varying doses during ovarian stimulation procedures.[10,11] It has been shown that HCG can upregulate or downregulate microRNA profiles in the endometrium. These hormones can also alter angiogenesis.[12,13] Tea, available in forms such as black, green, and oolong, is the second most widely consumed beverage after water. Green tea is derived from the leaves of the Camellia sinensis plant and is rich in polyphenols, particularly Epigallocatechin-3-Gallate (EGCG), which exhibits significant antioxidative, antimitotic, and antiangiogenic properties.[14,15] EGCG is the major bioactive component of green tea, representing approximately 2–13% in the dry green tea. Research indicates that EGCG exerts antiangiogenic properties by modulating VEGF signaling pathways.[16,17] EGCG can alter the microRNAs in various cells such as melanoma cells (microRNA-let-7b),[18] lung cancer cells (miR-210, hsa-miR-98-5p),[19] and hepatic cells (miR-33a and miR-122).[20] Thus, in the context of endometrial receptivity, changes in miRNA profiles due to EGCG may influence the expression of genes critical for implantation and placentation. For instance, miRNAs are known to regulate angiogenic factors and pathways that promote endometrial growth and function. Therefore, understanding how EGCG modulates miRNA expression may provide insights into its potential role in enhancing fertility and supporting successful implantation.

The increasing prevalence of ovarian stimulation in ART and the growing interest in natural compounds like EGCG for their potential therapeutic effects make it essential to investigate how these factors influence key determinants of endometrial receptivity for embryo implantation.

This study aims to fill this gap by examining the endometrial expression of miR-16-5p, VEGF protein levels, and CD31-positive cell count following EGCG administration in ovarian-stimulated mice. This research serves as a valuable model for studying human reproductive health and diseases, offering new insights into the molecular interactions that may affect fertility outcomes.

Methods and Materials

This is an experimental study. The design, data collection, and analysis were conducted from April 2022 to January 2024. Forty mature female and 20 adult male NMRI mice, aged 10–12 weeks and weighing 25–40 g, were used. Throughout the study, these mice were maintained under standard conditions, which included a constant room temperature of 22°C, a 12-hour light and 12-hour dark cycle, and unrestricted access to food and water. Four groups were established, with the HMG injection day designated as day 0 for all groups, which were randomly assigned: 1) Control group: No intervention was applied. 2) Ovarian stimulation (HMG/HCG) group: The mice received HMG (Shafayab Gostar Co-Iran) at a dose of 7.5 IU intraperitoneally (IP), and after 48 hours, HCG (Pooyesh Darou Co-Iran) at a dose of 7.5 IU IP was injected.[21] 3) EGCG group: The mice were treated with EGCG (Sigma-Aldrich, E4143) at a dose of 5 mg/kg IP at 0, 24, 48, and 72 hours after day 0.[22] 4) Ovarian stimulation + EGCG (HMG/HCG + EGCG) group: The mice received HMG 7.5 IU IP, and after 48 hours, HCG 7.5 IU IP was administered. Then, they were given EGCG at a dose of 1 mg/mouse IP daily for 4 days, starting from day 0. In each experimental group, two female mice and one male mouse were housed together overnight to facilitate mating. Successful mating was confirmed by the presence of sperms in a vaginal smear the following morning, which was designated as the first day of pregnancy. After 96 hours post HCG injection (just before implantation), the mice were euthanized under anesthesia. Blood was collected via cardiac puncture, and approximately one-third of the middle uterine horns were excised. The right uterine horns were fixed in 10% neutral buffered formalin for immunohistochemistry (IHC), while the left uterine samples were frozen in liquid nitrogen for real-time Polymerase Chain Reaction (PCR) analysis.[21]

Serum estradiol and progesterone levels were measured using ELISA. For serum isolation, the blood was centrifuged for 10 minutes at 4000 rpm; then an ELISA kit (Monobind –USA) with a sensitivity of 8.2 pg/mL was employed for detection of Serum estradiol, and progesterone was measured using a 0.1 ng/mL sensitivite ELISA kit (Monobind –USA). All assays were done based on the kit's instructions.[23] Gene expression analysis of miR-16-5p was performed using real-time PCR. Real-time PCR was employed to quantify the expression of miR-16-5p. Following the extraction of total RNA from the endometrium tissues using Trizol Reagent, sourced from Rojetecchnology Co. in Iran, RNA purity was assessed using the NanoDrop 2000, provided by Thermo Scientific, USA. cDNA synthesis was accomplished using the RT-PCR Pre-Mix Kit (Bio Fact-Korea). For real-time PCR, the Real-Time PCR Master Mix Kit (Bio Fact- Korea) was used along with specific miR-16-5p primers. U6 was used as internal reference. Data analysis was conducted using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used for real-time PCR were as follows: For miR-16-5p, the forward primer was GTTTGGTAGCAGCACGTAAATA, and the reverse primer was GTGCAGGGTCCGAGGT. For U6, the forward primer was CGCTTCGGCAGCACATATAC, and the reverse primer was AAATATGGAACGCTTCACGA.[24]

Immunohistochemical staining was used to assess VEGF protein expression and CD31-positive cell density in the endometrium. Uterine tissues were fixed in 10% neutral buffered formalin and then subjected to a series of dehydration steps using ethanol with increasing concentrations. The samples were embedded in paraffin and sliced into 4-µm sections. These sections were deparaffinized with xylene and rehydrated through a series of alcohol solutions with

decreasing concentrations. Following an overnight incubation at 4°C with a mouse monoclonal anti-VEGF primary antibody (Diagnostic Biosystems: PDM165), the sections were treated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (Protaqs: 300155400) according to the manufacturer's instructions. Harris' hematoxylin was used as a counterstain. The stained sections were then examined and photographed using a light microscope (Olympus Corporation, at 40 × magnification). The images were analyzed with ImageJ software (NIH, MD, USA).[25] For CD31 IHC, which identifies endothelial cells, the same procedure was followed, using the mouse monoclonal anti-CD31 antibody (Zytomed System: BMS044).[22]

In this study, statistical analyses were conducted using SPSS version 29 (IBM, New York, NY, USA). The one-way ANOVA test was utilized to assess differences among the groups. Data are expressed as mean ± standard deviation (SD). A p value of less than 0.05 was regarded as statistically significant.

Ethical considerations

The Ethics Committee of Isfahan University of Medical Sciences (Isfahan, Iran) granted approval for the current research, bearing the reference number IR.MUI.MED.REC.1400.606. All animal-related procedures strictly adhered to the guidelines outlined for the care and utilization of laboratory animals. The researchers ensured that the manuscript was free from plagiarism, reported the results honestly, and avoided data fabrication.

Results

Hormone analysis

ELISA results showed that EGCG reduced serum progesterone levels in both the EGCG and ovarian stimulation + EGCG groups. This reduction was statistically significant only in the EGCG group compared to controls ($p < 0.05$). The highest progesterone levels were observed in the HMG/HCG group, with a statistically significant difference when compared to the EGCG ($p < 0.05$) and ovarian stimulation + EGCG ($p < 0.05$) groups. However, there was no significant difference in progesterone levels between the EGCG group and the ovarian stimulation + EGCG group ($p > 0.05$) [Figure 1b, Table 1].

The lowest and highest concentrations of estradiol were observed in the EGCG group and the ovarian stimulation group, respectively, with both showing statistically significant differences compared to the control group ($p < 0.05$). ELISA results also indicated that EGCG administration in ovarian-stimulated mice significantly reduced estradiol levels in serum compared to the ovarian stimulation group ($p < 0.05$) [Figure 1c, Table 1].

MiR-16-5p expression

The real-time PCR method was used to assess miR-16-5p expression levels. As illustrated in Figure 1a, miR-16-5p was found to be upregulated in the HMG/HCG group relative to the control group ($p < 0.05$), with a fold change of 1.17. In contrast, the EGCG group exhibited the lowest miR-16-5p levels, and this reduction was statistically significant compared to the other groups (fold change of 0.72). No significant differences were observed between the ovarian stimulation + EGCG group and the control group ($p > 0.05$). However, miR-16-5p levels were significantly reduced in the ovarian stimulation + EGCG group compared to the ovarian stimulation group ($p < 0.05$).

Table 1: Serum progesterone and estrogen concentrations Mean (SD) in different study groups

	Estradiol concentration (pg/ml) Mean (SD*)	Progesterone concentration (ng/ml) Mean (SD*)
Control	19.81 (1.27)	12.16 (1.04)
HMG/HCG**	25.21 (1.13)	13.63 (1.13)
EGCG***	16.25 (0.72)	9.09 (0.61)
HMG/HCG** + EGCG***	21.24 (2.81)	10.74 (2.53)

*Standard Deviation. **Human Menopausal Gonadotropin/Human Chorionic Gonadotropin. ***Epigallocatechin Gallate

VEGF protein production

IHC results indicated that, compared with the control group (mean (SD) 6.70 (0.59)), the VEGF protein was upregulated in the ovarian stimulation group (mean (SD) 7.82 (0.49)) ($p < 0.05$). The groups receiving EGCG as part of their treatment regimen (EGCG group and ovarian stimulation + EGCG group) exhibited a decrease in the expression of VEGF protein and the lowest level of VEGF protein was observed in the EGCG group, which was significantly different from the control group ($p < 0.05$). However, there were no statistically significant differences between the ovarian stimulation + EGCG group and the control group, as well as between the ovarian stimulation + EGCG group (mean (SD) 6.39 (0.54)) and the EGCG group ($p > 0.05$). The injection of EGCG into ovarian-stimulated mice (ovarian stimulation + EGCG group) caused a significant decrease in protein expression compared to the ovarian stimulation group ($p < 0.05$), Figure 2a.

Endothelial cell density

IHC for CD31, a marker for endothelial cells, indicated that the number of CD31-positive cells was significantly higher in the ovarian stimulation group (mean (SD) 21.10 (1.52)) compared to the control group (mean (SD) 18.50 (1.58)) ($p < 0.05$). No significant difference in CD31-positive cell counts was found between the ovarian stimulation + EGCG group and the control group ($p > 0.05$). The EGCG group (mean (SD) 12.90 (1.66)) exhibited the lowest number of CD31-positive cells. This reduction was statistically significant compared to the control group ($p < 0.05$), the ovarian stimulation group ($p < 0.05$), and the ovarian stimulation + EGCG group ($p < 0.05$), as shown in Figure 2b.

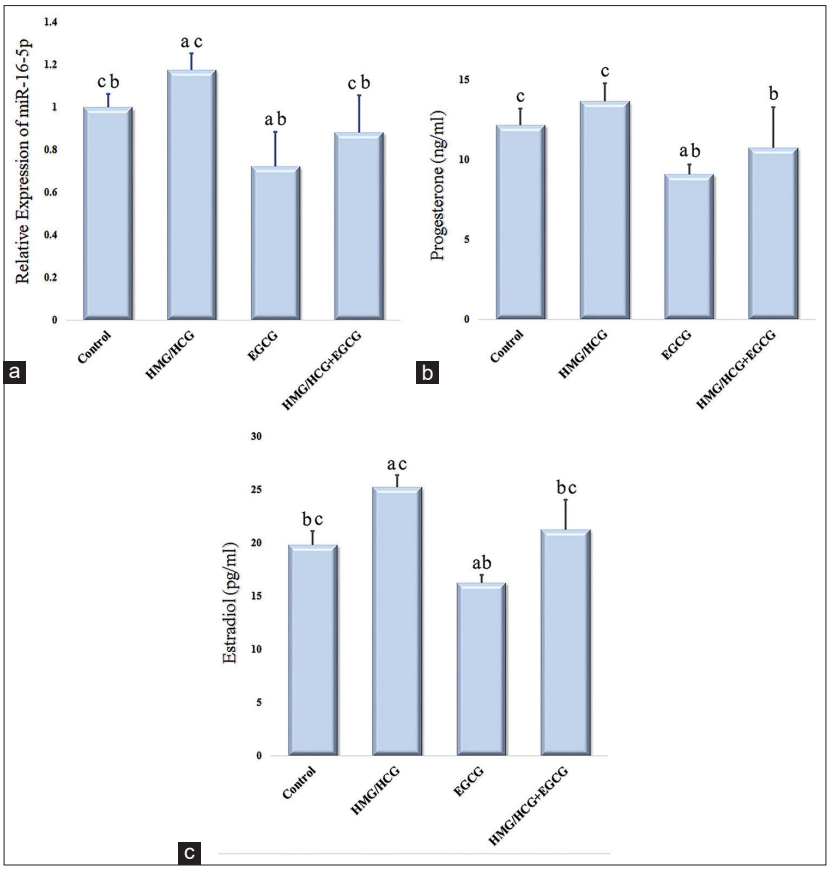

Figure 1: Endometrial expression of miR-16-5p (a) serum concentrations of progesterone (b) estradiol (c). Letters a, b, and c denote statistically significant differences compared with the control group, HMG/HCG group, and EGCG group, respectively. Data are presented as mean (SD), with statistical significance set at $p < 0.05$

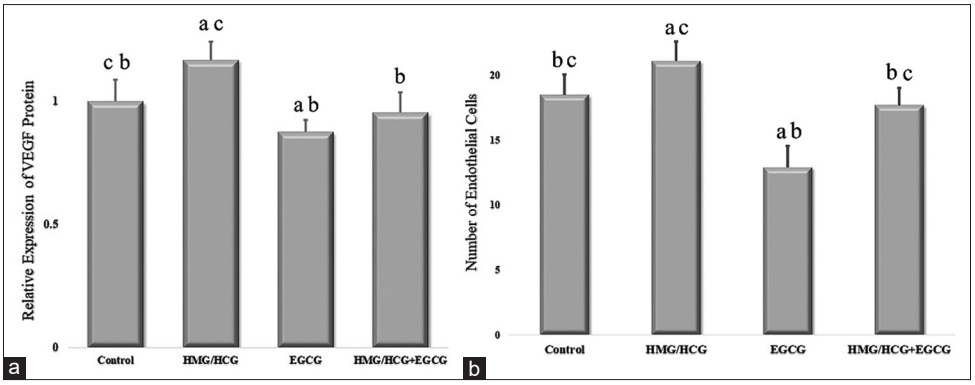

Figure 2: Quantitative analysis of VEGF protein expression (a) and endothelial cell density in the endometrium (b). Letters a, b, and c represent statistically significant differences compared to the control group, HMG/HCG group, and EGCG group, respectively. Data are shown as mean (SD), with statistical significance defined as $p < 0.05$

Discussion

Considering the significance of angiogenesis in the receptivity of the endometrium during the implantation window, angiomiRs may stand out as potentially vital determinants in female fertility. These are a subclass of microRNAs that regulate the expression of genes involved in angiogenesis at the post-transcriptional level.[3,26]

In this study, the administration of HMG/HCG, which directly influences the same biological pathways as FSH and LH, was found to upregulate the endometrial expression of miR-16-5p compared to the control group. On the other hand, the groups receiving EGCG as part of their treatment regimen exhibited a decrease in the expression of this miRNA.

Previous research strongly indicates that EGCG has the capacity to modulate the expression of multiple microRNAs. Notable changes in the expression levels of microRNAs, including hsa-miR-125a-3p, hsa-miR-15b-3p,

hsa-miR-548av-3p, hsa-miR-125a-3p, hsa-miR-500a-3p, hsa-miR-7706, and hsa-miR-15b-3p, have been observed in non-small-cell lung cancer cells following treatment with EGCG.[27] Green tea and EGCG can affect the expression of let-7b. EGCG has also been shown to upregulate the expression of miR-210, which plays a crucial role in regulating AKT, MAP kinases, and the cell cycle.[18,28] However, in previous studies, the expression of miR-16 and miR-17 in the endometrium has been determined following ovulation stimulation, but it appears that no study has investigated the effect of EGCG on the endometrial expression of angiomiR-16.[21]

In our study, HMG/HCG administration increased the serum concentrations of progesterone and estradiol. Elevated levels of these hormones in the serum following ovarian stimulation have been demonstrated previously. In stimulated cycles, these hormones induce molecular and gene expression changes that affect endometrial receptivity during the implantation window.[25,29] Additionally, in the current study, the injection of EGCG (both in the EGCG group and the ovarian stimulation + EGCG group) led to a decrease in the serum concentrations of these hormones.

Research indicates that estrogen and progesterone can affect microRNA synthesis. MicroRNAs are transcribed by RNA polymerase II, resulting in the production of primary miRNA (Pri-miRNA). The pri-miRNA is then processed by Drosha, a nuclear RNase III enzyme, in conjunction with its cofactor, DGCR8. This processing cleaves the pri-miRNA to produce pre-miRNA.[2,30] Studies have demonstrated that high estrogen receptor expression is associated with high expression of Drosha and DGCR8. Other studies suggested that estrogen alone can increase DGCR8 and had no effect on Drosha, whereas progesterone and HCG do not significantly impact on Drosha and DGCR8 expression.[31,32] The pre-miRNA is exported to the cytoplasm by exportin-5 (EP5), where it undergoes processing by Dicer, generating double-strand miRNA. Estrogen and progesterone have been shown to enhance the expression of Exportin-5 and Dicer-1, whereas HCG does not influence the expression of these two molecules.[33,34] No studies have been identified to elucidate the potential mechanisms by which EGCG reduces expression of angiomir or miRNAs in general. The duplex miRNA/miRNA is conveyed to the RNA-induced silencing complex (RISC) for conversion into a mature, single-stranded form to contribute significantly to gene regulation.[35]

In the present study, we observed a notable increase in VEGF protein levels in the HMG/HCG group compared to the control group, as measured by IHC. In contrast, the EGCG group exhibited a significant decrease in VEGF protein levels.

VEGF is present in the endometrial luminal and glandular epithelium, as well as in the stromal cells. It has been demonstrated that both endometrial epithelial and stromal cells express HCG receptors, and the binding of HCG with these receptors results in increased VEGF expression.[36,37]

EGCG influences endometrial angiogenesis-related factors such as growth factors VEGF and VEGFR2.[38] Furthermore, EGCG can target specific receptors, including the insulin-like growth factor-1 (IGF-1) receptor in stromal and luminal and glandular endometrial epithelium,[39,40] the FAS receptor (CD95) in stromal cells.[41,42] It can also inhibit the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptors (VEGFRs).[43,44] Interference with these receptors by EGCG could lead to decreased VEGF levels in the endometrium.

The exact molecular mechanism underlying the alteration in VEGF protein expression following the administration of HMG/HCG and EGCG is not completely clear. It is suggested that microRNAs may play a crucial role in this process. It has been reported that VEGF is a direct target for miR-16-5p (http://mirtarbase.mbc.nctu.edu.tw/php/index.php). In the canonical and original model of miRNA function, the RISC typically binds to the 3′ untranslated region (UTR) of target mRNAs, initiating inhibition of translation;[45] however, it is now revealed that miRNAs have the potential to bind to and interact with various sites on the target mRNAs, such as 5′ UTR and coding sequence (CDS) region. Both negative and positive regulatory effects of miR-16-5p on VEGF protein expression have been shown.[21]

Additionally, lncRNA-miRNA–mRNA network is probably involved in these results. It is also reported that some lncRNAs that are target for miR-16-5p acted as competing endogenous RNAs (ceRNA) and competed with mRNA of other targets for binding to miR-16-5p, competing endogenous RNA hypothesis.[46] However, more studies are required to reveal the role ceRNA in context of endometrium.

Furthermore, the expression of a single mRNA can be influenced by several miRNAs, and each miRNA can target multiple mRNAs. According to the miRWalk 2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), in mice, VEGF is targeted by mumiR-150-5p, mmu-miR-340-5p, mmu-miR-466f-3p, mmu-miR-329-3p, and others. Studies have indicated that during non-natural cycles, the expression of certain miRNAs targeting VEGF, including miR-34-5p, miR-423-5p, miR-34b, miR-503, miR-520g, miR-369-3p, and miR-186, is altered.[47,48] On the other hand, Wnt, Bcl2, Jagged1, and Fgf2[49,50] are additional targets for miR-16-5p, that are associated with the VEGF protein-related pathways, suggesting an indirect alteration of VEGF protein expression by miR-16-5p.

In the present study, a notable increase in CD31-positive cell density was observed in the HMG/HCG group compared to the control group, and the EGCG group exhibited a significant decrease in CD31-positive cell expression. It has been demonstrated that endothelial cell

proliferation is affected by the VEGF/VEGFR system and the presence of VEGF is essential for endometrial receptivity by promoting angiogenesis.[51] The binding of VEGF to its receptor initiates intracellular signaling pathways that play a critical role in endothelial biology. On the other hand, it has been shown that endothelial cell proliferation can be increased by the activation of HCG in endometrial cells.[52] Conversely, during implantation window, EGCG may induce apoptosis and inhibits the proliferation and migration of endometrial endothelial cells by regulating the expression of genes associated with apoptosis and autophagy. More studies are needed.

The maximum and minimum levels of miR-16-5p, VEGF protein, and endothelial cell density have been seen in the HMG/HCG group and EGCG group, respectively. Levels of steroid hormones could potentially alter molecular patterns in the endometrium, impacting uterine receptivity. This alteration might involve changes in miRNA profiles and VEGF protein expression.[25,29] We suggest that these observed outcomes may be result from changes in steroid hormone levels following ovarian stimulation, EGCG administration, or a combination of these factors. As a preliminary study, it was not ethically possible to conduct this research with human samples, which may be considered a limitation. Therefore, more studies are needed to pave the way for future research involving human samples.

Conclusion

The findings of the current research suggest that ovarian stimulation and an antiangiogenic factor (EGCG) influence the endometrial expression of miR-16-5p. These miRNA, known as angiomiR, may impact endometrial receptivity during the implantation window through changes in VEGF protein expression, subsequently leading to increased proliferation of endothelial cells, or via other distinct biological pathways.

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Conflicts of interest

Nothing to declare.

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