Evaluation of Endometrial Angiogenesis in Mice Uterus Before Implantation in Natural Cycles Followed by Use of Human Menopausal Gonadotropin - Human Chorionic Gonadotropin Drugs and Epigallocatechin Gallate

Abstract

Background: Angiogenesis plays a major role in endometrial receptivity and thickening of the endometrium immediately before implantation. The aim of the present work was to evaluate the antiangiogenic properties of epigallocatechin-3-gallate (EGCG) from green tea in angiogenesis of endometrium. **Materials and Methods:** In this study, forty adult female NMARI mice randomly divided into four groups. Control group received vehicle; human menopausal gonadotropin/human chorionic gonadotropin (HMG/HCG) group received 7.5 IU HMG intraperitoneal (IP) and 48 h later 7.5 IU HCG was injected (IP) for ovarian stimulation; HMG/HCG + EGCG group received HMG and HCG in the same manner as the previous group and also received 5 mg/kg EGCG at 0, 24, 48, and 72 h after injection of HMG; and the group EGCG received 5 mg/kg EGCG. A male mouse was kept with two female animals in the same cage for mating. Mice were dissected 96 h after administration of HMG (immediately before implantation) and tissue processing was carried out for the uterine specimens. CD31-positive cells were counted by use of histological and immunohistochemical methods. **Results:** Angiogenesis in EGCG-treated group was less than that of control and gonadotropin group ($P < 0.05$). The number of endothelial cells was counted by CD31 marker under a light microscope and showed significant differences between all groups ($P < 0.05$). **Conclusion:** EGCG significantly inhibited the angiogenesis in endometrium (in natural cycles) through antiangiogenic effects.

Keywords: *Angiogenesis, epigallocatechin gallate, human menopausal gonadotropin/human chorionic gonadotropin, implantation*

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Introduction

Neovascularization occurs in two forms: vasculogenesis and angiogenesis. Vasculogenesis is the process whereby hemangioblasts differentiate into blood cells, and mature endothelial cells and new blood vessel is formed.[1] On the other hand, angiogenesis is the formation of new blood vessel from preexisting vascular networks.[2] Angiogenesis is divided into two components: physiological and nonphysiological or pathological angiogenesis. Physiological angiogenesis occurs during the process of wound healing and tissue repair, during the formation of ovarian corpus luteum, and placental development establishing pregnancy. Pathological angiogenesis occurs during rheumatoid arthritis, diabetic retinopathy, psoriasis, endometriosis, and tumor growth and metastasis.[3] Endometriosis is a chronic disorder characterized by the implantation of endometrial glands and stroma outside the uterine cavity. Despite different hypotheses regarding the pathogenesis of endometriosis, it is known that angiogenesis plays an important role in the growth and survival of endometriotic lesions and endometriosis is an angiogenesis-dependent disorder.[4] Antiangiogenesis therapy offers a new chance for the treatment of endometriosis.[5] Bezafibrate, as pan-PPAR agonist, restores angiogenesis in hindlimb ischemic diabetic animals and may be beneficial for the prevention or treatment of peripheral artery disease in diabetic subjects.[6]

Globally, tea is the second most popular beverage after water.[7] It is consumed in different parts of the world as black, green, and oolong tea. Green tea is

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prepared from the leaves of the tea plant called *Camellia sinensis,* and the impact of its consumption on several diseases and populations have been reported in clinical studies and laboratory animals.[8] Green tea, which includes 20% of the world's tea consumption, is made by steaming or pan-frying the leaves and then drying them to inactivate enzymes and prevent the oxidation of the tea's components.[9] These processes preserve the tea polyphenols known as catechins which account for 30%–40% of the dry weight.[10] The main catechins found in green tea include epigallocatechin-3-gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and epicatechin,[11] gallocatechin gallate (GCG), catechin gallate, and catechin.[12] EGCG is the most abundant and active catechin in green tea, which accounts for 50%–80% of the catechin content.[13] The polyphenols found in green tea, especially epigallocatechin-3-gallate (EGCG), have strong antioxidative, antimitotic, and antiangiogenic properties.[14] The antiangiogenic activity of EGCG has been widely proved *in vitro* and *in vivo*.[15] In previous studies, it was reported that EGCG suppressed the angiogenesis signaling pathway and inhibited neovascularization and the growth of experimental endometriosis in mice.[16,17]

Due to the similarity in nature and histologic features of endometrial and endometriosis lesions and considering the fact that angiogenesis plays a major role in endometrial receptivity and thickening of the endometrium immediately before implantation, it is critically important to investigate the study that shows whether the contents of green tea can inhibit angiogenesis and subsequent reduction of implantation. The endometrium is one of the most interesting tissues in the human body.[18] The human endometrium endures a complex series of organized proliferative and secretory alterations in each menstrual cycle, and shows only a short period of receptivity, known as the "window of implantation."[19] All these are affected by ovarian hormones, estrogen, and progesterone.[20] Experts utilized gonadotropin (human menopausal gonadotropin [HMG]/human chorionic gonadotropin [HCG]) to induce ovarian follicle development and luteinizing hormone surge in *in vitro* fertilization and intracytoplasmic sperm injection procedures.

In the luteal phase, endometrium provided the conditions for implantation.[21] This study aims to survey the effect of gonadotropins and EGCG on angiogenesis in mice endometrium. Platelet endothelial cell adhesion molecule or CD31 is a new marker for the detection of angiovasculogenic activity express in endothelial cells with high levels of angiogenic activity. Due to ethical considerations, animal model was utilized for the preparation of tissue sections. In a review of literature, no study has been carried out on the effect of EGCG on endometrial angiogenesis.

Materials and Methods

Animals

For this study, forty adult female NMRI mice (3 months old) and twenty adult male NMRI mice, weighing about 25–30 g were housed in animal house of Medical School of Isfahan University of Medical Sciences within a temperature-controlled environment on a 12 h/12 h light–dark cycle while water and standard animal food diet were provided *ad libitum*. Female mice were randomly divided into four groups of ten as a control group, receiving HMG/HCG and the recipient of the EGCG with gonadotropin (HMG/HCG) and receiving EGCG group. In all groups, two female mice were placed with a male mouse in a cage for mating for a day.

Drugs

HMG (Ferring, Germany) and HCG (Ferring, Germany) were utilized to artificially induce the growth of ovarian follicles and ovulation. Moreover, EGCG (Sigma, USA) as an antiangiogenic agent was utilized in target groups.

Study interventions

No intervention was done in the control group. Within the groups receiving gonadotropin, the 7.5 IU HMG was injected (intraperitoneally [IP]) and 48 h after injection of HMG, 7.5 IU HCG was injected for ovarian stimulation. In the group receiving the drug substance, at 0, 24, 48, and 72 h after HMG injection, 5 mg per kg EGCG (IP) was injected. The respective doses of EGCG were selected as they had been proven to have significant antiangiogenic effects in mice *in vivo*.[22] Thereafter, in the whole group, two female mice with a male mouse were placed in a cage for mating. Mice were sacrificed using anesthesia 96 h after HMG administration (immediately before implantation) and 1.3 middle uterus was washed with culture medium.

Histology staining

The uterus of the mice containing blastocyst was sampled. The samples were buffered in 10% formalin for fixation. Thereafter, they underwent stages of tissue processing with ascending grades of alcohol, cleared with xylol, and finally, embedded in paraffin. The samples with 4-μ tissue sections using microtome serial sections were stained with periodic acid-Schiff (PAS) and analyzed utilizing an optical microscope.

Immunohistochemistry staining

Endometrial samples were fixed by 10% Buffered Formalin (Sigma, USA) for 24 h and were subjected to tissue processing with ascending grade of alcohol and transparency in Xylene (Sigma, USA). Finally, they were embedded in paraffin, and 4-μ tissue sections using microtome serial sections (slice vector) were prepared. The immunohistochemistry (IHC) method was utilized to detect angiogenesis in endothelial cells. In this method, the 4-μ

thick slices were removed and placed on slides previously covered with poly-L-lysine in all groups. They were stained by IHC. The basis of this method is that in the first level, antigen binds to the primary antibody, anti-CD31 antibody (AbCam, UK), and then the secondary antibody binds to the primary antibody as its antigen for the second level. Antibody-labeled and antibody complex can be shown using the enzyme substrate. Due to the reaction of peroxidase enzyme with diaminobenzidine (DAB); DAB is utilized as a peroxidase substrate, and a dark brown product is formed.

Statistical analysis

The different field in every mice and the group were randomly determined using microscopes connected to computer with ×40 magnification and Motic Images Advanced 3.2 software (Motic China Group Co., Ltd), and then CD31 positive cell count was performed. Finally, groups were compared in terms of the number of endothelial cells using software SPSS (version 20, SPSS Inc. Chicago, USA). and the Kruskal–Wallis test. As mentioned above, a simple random sampling method was used and the level of statistical significance was set at $P < 0.05$.

Results

Morphometric and histomorphologic study

In microscopic observation of the uterus in mice; perimetrium, myometrium, endometrium with specified thickness can be clearly seen. Endometrium characterized by myometrium to top of the epithelial cell has different thickness in a different part. In some parts; endometrial thickness which contains stroma, uterine glands, and luminal epithelium is found several times in some part with just a row of epithelium and small amount of stroma. Observation of glandular cells, lumen of the uterus and luminal epithelium folds with larger magnification (×40) revealed that the nucleus of glandular and luminal cells is located in the basal area with abundant extracellular matrix between the stromal cells. All these observations revealed that dropsically endometrium is formed in preimplantation or endometrium window stage [Figure 1]. The glandular epithelium cells in the form of short cylindrical lumen-containing secretions can be seen. The uterine luminal epithelium cells in the form of a cylinder with apical secretory granules and basement membrane is visible [Figure 1].

Immunohistochemistry study

Optical microscopy studies revealed that long columnar luminal epithelial cells with many PAS positive granules are placed mainly in the basal cell surface (in the subnuclear area) in the control group [Figure 2]. In group gonadotropin [Figure 3], the long columnar luminal epithelial cells with many PAS positive granules were in supranuclear and basal area. In the other two groups, EGCG [Figure 4], and gonadotropin + EGCG [Figure 5],
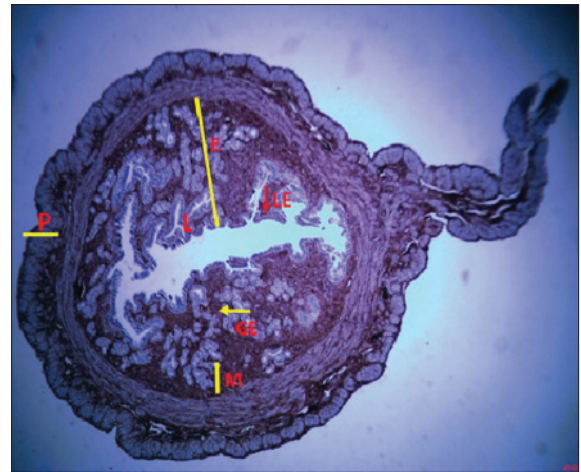

Figure 1: Histological morphology in uterus (luteal phase in all groups). P: Perimetrium, M: Myometrium, E: Endometrium, L: Lumen, LE: Luminal epithelium, GE: Glandular endometrium
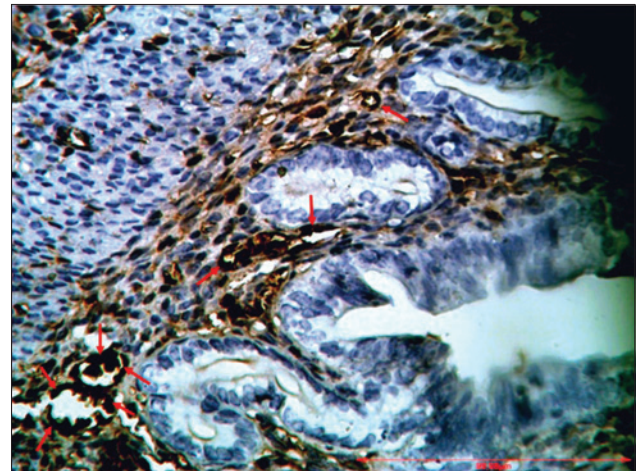

Figure 2: CD31-positive cells in control group (×400), endothelial cells are shown with arrows

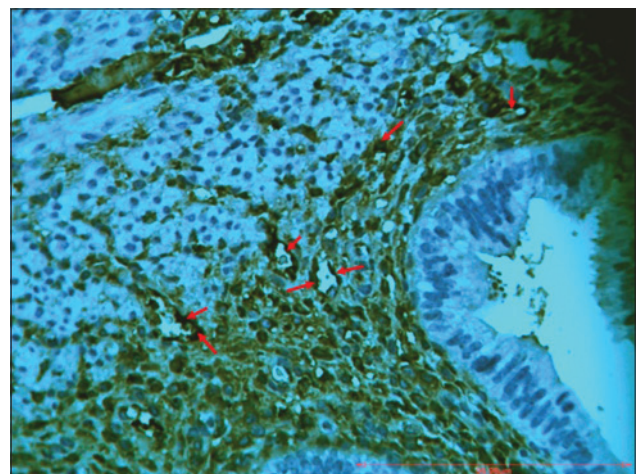**Figure 3: CD31-positive cells in EGCG group (×400), endothelial cells are shown with arrows**

cells were seen as columnar and PAS positive granules in the supranuclear and basal area (in the subnuclear area). In all groups, nucleus was central
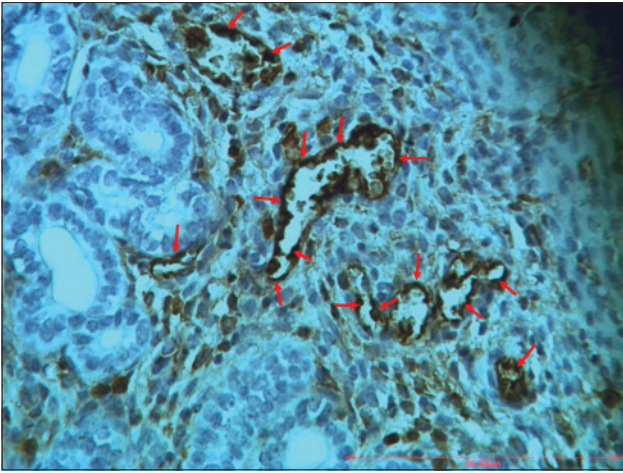
Figure 4: CD31-positive cells in human menopausal gonadotropin/human chorionic gonadotropin group (×400), endothelial cells are shown with arrows

Figure 5: CD31-positive cells in human menopausal gonadotropin/human chorionic gonadotropin + EGCG group (×400), endothelial cells are shown with arrows

and seemed to be vacuolated, which represents the luteal phase of endometrium.

Moreover, the results from Kruskal–Wallis test reveal that the difference between the mean of CD31 positive cells in all groups was significant [$P < 0.05$, Figure 6].

According to our findings, the mean of CD31 positive cells were 31.85 ± 3.53 in control group, 43.08 ± 3.96 in gonadotropin group, 15.90 ± 3.83 in gonadotropin + EGCG group, and 19.53 ± 4.72 in EGCG group. The results showed that angiogenesis parameter can be affected by exogenous factors such as gonadotropins and EGCG. The utilization of gonadotropins in the ovulation-stimulating process increased angiogenesis in this group in comparison with control group. In the other group, the use of gonadotropins + EGCG led to a reduction in the angiogenesis when compared with gonadotropins and control group. According to these results, the inhibitory role of EGCG and activating role of gonadotropins on angiogenesis can be inferred.

Discussion

The results of this study indicate that EGCG with a dose of 5 mg/kg after different durations (before implantation) in receiving EGCG groups (EGCG and EGCG + gonadotropin groups) inhibit angiogenesis in uterus of mice in comparison with nonreceiving groups (control and gonadotropin groups). The utilization of gonadotropins in the ovulation-stimulating process increased angiogenesis in this group in comparison with control group [Figures 1 and 2]. In the other group, the use of gonadotropins + EGCG led to a reduction in the angiogenesis when compared with gonadotropins group. The comparison of gonadotropins + EGCG and EGCG group demonstrated that angiogenesis in gonadotropins + EGCG is lower than EGCG group. Our results had many similarities with the results of studies of EGCG on endometriosis lesions.[16] Optical microscopy studies revealed that PAS
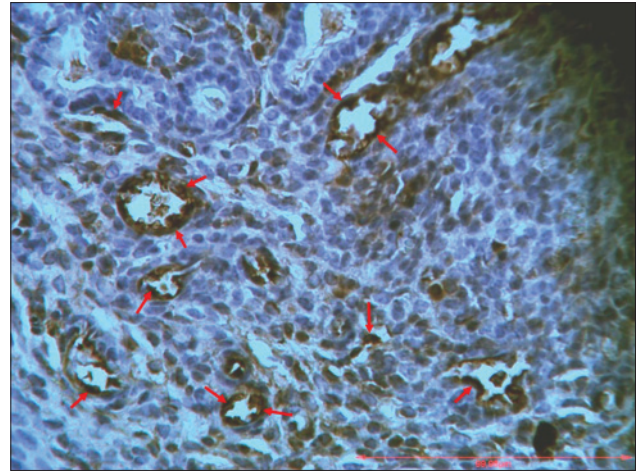
positive granules are mainly placed in the basal cell surface in the control group, while PAS positive granules are placed in supranuclear and subnuclear area in other groups.

Xu *et al.* in 2009 reported that EGCG with dose of 50 mg/kg IP has antiangiogenic effects on an experimental endometriosis mouse model.[16] Although Nakae, *et al.* in 2008 demonstrated the dose-dependent antiangiogenic effects, they proved that EGCG at the lower dose of 5 mg/kg IP also had the same result.[22] Since all previous studies were on endometriosis and no study has been carried out on the effect of EGCG on endometrium yet; therefore, the minimum dose was used (5 mg/kg) as the first study on uterine endometrium.

In general, angiogenesis is the growth of new vascular capillary channels from preexisting vessels. Angiogenesis is regulated by the balance between a huge number of pro- and anti-angiogenic factors.[23] Three families of receptor protein-tyrosine kinases are involved in vasculogenesis and angiogenesis. The vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) family is the most important regulator of vascular development. The angiopoietin-Tie system controls vessel maturation and quiescence[24] while the eph-Ephrin system controls positional conduction cues and arterio-venous asymmetry.[25]

Lee, *et al.* in 2014 reported that epigalloccatechin-3-gallate inhibits neovascularization and vascular permeability through the suppression of MMP-9 and VEGF activation.[26]

Cerezo-Guisado, *et al.* in 2015 reported that EGCG-induced cell death is partially blocked by inhibiting Akt, ERK1/2 activity.[27] PI3K/Akt and MAPK/ERK pathways regulate HIF-1 α protein synthesis which strongly activates VEGF expression.[2] Moreover, the only catechin that can inhibit VEGF binding to its receptor is EGCG.[28]
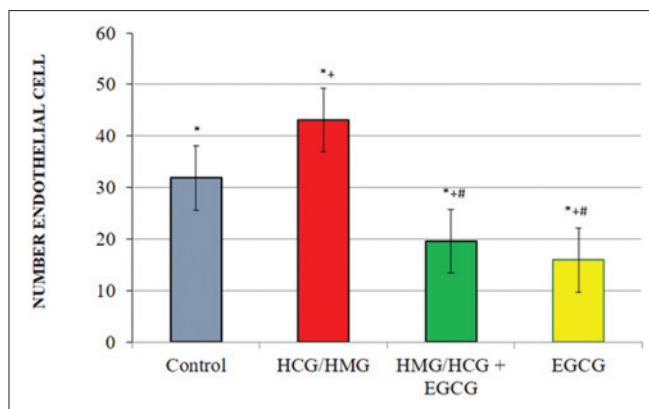
Figure 6: Graphical comparison of four groups with the mean ± standard deviation that shows significant difference between the four groups. As indicated, there is a significant difference between the four groups ($P < 0.05$). These symbols demonstrated different group (*+#). (*) Significant difference between control group with EGCG group, menopausal gonadotropin/human chorionic gonadotropin group and human menopausal gonadotropin/human chorionic gonadotropin + EGCG group. (+) Significant difference between EGCG group with menopausal gonadotropin/human chorionic gonadotropin group and human menopausal gonadotropin/human chorionic gonadotropin + EGCG group. (#) Significant difference between menopausal gonadotropin/human chorionic gonadotropin group with human menopausal gonadotropin/human chorionic gonadotropin + EGCG group

Deng, *et al.* in 2013 concluded that EGCG leads to a reduction in interstitial fluid pressure and hypoxia and improve chemotherapy efficacy through rebalance of Ang-1 and Ang-2.[29] Ephrin (Eph) is a third critical mediator of angiogenesis.[30] Tang, *et al.* in 2007 indicated that activation of ERK-1/2 plays an essential role in ephrin-A1-mediated cell migration. EGCG inhibited ephrin-A1-mediated endothelial migration and angiogenesis.[31] On the other hand, EGCG can arrest cell cycle[32] and induce apoptosis[33] and cell death.[34]

In summary, green tea inhibits angiogenesis through several mechanisms: cell cycle arrest,[32] induction of apoptosis,[35] reduced expression of VEGF,[2] inhibition of VEGF/VEGFR,[28] inhibition of phosphorylation of VEGFR. Since EGCG has been reported to inhibit angiogenesis in endometriosis,[16] we decided to investigate the effects of EGCG on angiogenesis of endometrium.

According to the previous study, EGCG significantly inhibited the development of endometriosis through antiangiogenic effects.[16] It is well knowing that angiogenesis plays a major role in endometrial maturation and thickening of the endometrium before implantation. In this study, it was concluded that green tea can inhibit angiogenesis and endometrial implantation which is followed by a decline. Since no study about molecular pathway of EGCG on endothelial cells of endometrium has been reported, it is therefore recommended that more studies should be carried out.

Conclusion

Angiogenesis plays an essential role in endometrial thickness, its increase or decrease can be effective in fertility and infertility. Therefore, our study suggested that because EGCG has a significant inhibitory effect on the development of endometrium, women who are planning for pregnancy should avoid it due to the severity of its limitations.

Conflicts of interest

There are no conflicts of interest.

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