Enhanced expression of vascular endothelial growth factor and increased microvascular density in women with endometrial hyperplasia: a possible relationship with uterine natural killer cells

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Abstract
This case-control study aimed to investigate the expression of natural killer cells (NKCs) and the integrated optical density (IOD) of vascular endothelial growth factor (VEGF) and to quantify microvascular density (MVD) in endometrial biopsies from women with endometrial hyperplasia (EH) relative to normal subjects. Histological data from four groups were analyzed. The study population included 30 women with simple EH without atypia, 25 patients with complex EH without atypia, 25 with complex EH with atypia and 25 healthy women with non-hyperplastic endometrium (control group). Paraffin sections were immunostained with antibodies against CD56, VEGF-A and CD34 using an Avidin–Biotin–Peroxidase technique. The evaluation of NKC density and IOD of VEGF expression and measurement of MVD were performed using light microscopy examination and image analysis techniques. Increased numbers of NKCs were documented in cases of complex EH with atypia compared with the other groups (p<0.001). The number of NKCs was lower in cases of hyperplasia without atypia compared with the controls, but the difference was not significant. The IOD of VEGF-A and MVD increased significantly with progression from the non-hyperplastic endometrium through the three groups of EH (p<0.001). We observed a significant correlation between the MVD and the IOD of VEGF-A in the studied groups (r=0.434; p<0.001). Additionally, NKCs density was correlated significantly with IOD of VEGF-A (r=0.661; p<0.001) and with the MVD (r=0.473; p<0.001). These results suggest that NKC-count, IOD of VEGF and endometrial MVD are all related to the histological changes of the endometrium and that endometrial hyperplasia exhibits distinct immunological backgrounds in the context of NKC infiltration and VEGF production.

Keywords: NKCs, VEGF, MVD, integrated optical density, endometrial hyperplasia.

Introduction
The broad pathologic diagnosis of endometrial hyperplasia covers a spectrum of entities. At one end of the spectrum is simple hyperplasia without atypia, highly reversible proliferation with slightly crowded and dilated endometrial glands, characteristic of the response to mild estrogenic stimulation in the absence of progestin influence. At the other end of the spectrum is complex atypical hyperplasia, which exhibits nearly no stroma and markedly abnormal nuclei and resembles well-differentiated endometrial adenocarcinoma in biological and morphological respects [1].

The uterus produces leukocytes and cytokines that affect uterine cell proliferation, differentiation, and apoptosis [2]. Menstruation occurs because of an inflammatory process during which leukocytes infiltrate the endometrial tissue and produce proteases, chemokines, and cytokines. Uterine natural killer constitute up to 70% of the decidual leukocyte population in the first half of pregnancy and are considered to be an important source of regulatory growth factors and cytokines. In this respect, the balance between immunity and tolerance is important to maintain immune homeostasis, thus justifying the many mechanisms involved in keeping the immune response under control, including the activity of the natural killer and T-regulatory cells [3, 4].

Angiogenesis plays an important role in endometrial growth and is essentially needed for regenerative, hyperplastic and neoplastic conditions as well as tumor growth and metastasis because it allows the tissue to increase in size beyond the constraints of its original blood supply and permits tumor cell metastasis [5]. Endothelial cells of tumor-associated neovasculature can proliferate 20–2000 times more rapidly than endothelial cells of normal tissues. Intratumoral microvessel density determined by staining endothelial antigens on histological sections may be used as a quantitative measure of angiogenesis. Small blood vessels as well as capillaries can be detected on immunohistochemistry with a range of specific antigens [6].

Although many growth factors can induce endometrial angiogenesis, vascular endothelial growth factor (VEGF) is essential in controlling endometrial angiogenesis and has been considered as a key factor in the development of abnormal uterine bleeding [7]. VEGF modulates endometrial vascular permeability and angiogenesis as a paracrine factor during the menstrual cycle [8] and enhances endo-
metrial vascular permeability and angiogenesis by inducing the expression of its receptors in the local endometrial environment [9].

The aim of the present study is to evaluate the frequency of NKCs and the integrated optical density (IOD) of VEGF expression and to quantify the microvascular density in women with endometrial hyperplasia compared with normal subjects and to verify their possible association with the histological changes of the endometrium.

Materials and Methods

Case selection and tissue collection

This case-control study was performed on endometrial biopsies from 105 women attending Taibah University Teaching Hospital’s Al-Madinah Maternity and Children Hospital and Ohud Hospital, Saudi Arabia, between October 2013 and September 2014. The study was approved by the Local Medical and Health Sciences Research Committee, and all women gave informed consent before enrolment in this study.

The participants were classified into four groups according to the histopathological findings of the endometrium. The control group consisted of 25 women undergoing operations for benign reasons unrelated to endometrial dysfunction (e.g., leiomyoma, cervical dysplasia, and uterine prolapse) who were diagnosed as normal non-hyperplastic endometrium. The second group included 30 women diagnosed with simple endometrial hyperplasia without atypia. The third group included 25 women with complex endometrial hyperplasia without atypia. The fourth group included 25 women diagnosed with complex endometrial hyperplasia with atypia.

The included women presented with abnormal uterine bleeding or for infertility evaluation. Samples of normal and hyperplastic endometrial tissue were obtained at diagnostic curettage or at hysterectomy. None of the women had received preoperative steroid therapy. At the time of diagnostic curettage or hysterectomy, the endometrial tissue was immediately fixed in 10 mM citrate-buffered formalin and was processed for either histological or immunohistochemical study.

Histological and immunohistochemical processing

The endometrial tissues were immediately washed twice with phosphate-buffered saline (PBS) containing 0.1% sodium and fixed in 10% neutral buffered formalin or in Bouin’s solution at 4°C overnight, then dehydrated and embedded in paraffin. Tissues were divided into two blocks per patient and embedded in paraffin wax. Tissue sections (5 μm) were cut and stained with Hematoxylin–Eosin (HE) for general histological examination. Serial sections (3 μm) were deparaffinized, followed by endogenous peroxidase quenching with 3% H₂O₂/methanol. Non-specific binding was reduced by incubation with 5% bovine serum albumin. Heat retrieval in 0.1 M citrate buffer was used for the detection of CD56 and VEGF, whereas antigen retrieval in trypsin was used for the detection of CD34. The characteristics of the primary antibodies used are described in Table 1.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>Clone No.</th>
<th>Species</th>
<th>Catalog No.</th>
<th>Pretreatment</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56</td>
<td>Spring Bioscience</td>
<td>SPM128</td>
<td>Human</td>
<td>E1371</td>
<td>Citrate HR</td>
<td>Ready to use</td>
</tr>
<tr>
<td>VEGF</td>
<td>Spring Bioscience</td>
<td>SP28</td>
<td>Human</td>
<td>M3281</td>
<td>Citrate HR</td>
<td>Ready to use</td>
</tr>
<tr>
<td>CD34</td>
<td>Spring Bioscience</td>
<td>QBE310/10</td>
<td>Human</td>
<td>E1281</td>
<td>0.1% Trypsin</td>
<td>Ready to use</td>
</tr>
</tbody>
</table>

Primary antibodies were applied overnight at 4°C. The sections were then washed in PBS and incubated with biotinylated anti-mouse IgG (LSAB™2 Kit; Dako), followed by washing in PBS and then incubation with Avidin–Biotin–Peroxidase complex solution (LSAB™2 Kit; Dako). The antibody bound to sections was visualized by treating with 0.05% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemicals Co., St. Louis, MO, USA) in 10 mM Tris-buffered saline. Then, the sections were stained by Mayer’s Hematoxylin as a counter stain. For negative controls, incubation was performed with a non-specific IgG antibody at the same concentration as the primary antibody [10].

Evaluation of immunohistochemical staining

Tissue sections were examined using an optical microscope at 4× and 10× magnification for the initial screening. Images were digitalized in a 512×512-pixel matrix using a color video camera (digital camera CH-9435 DFC 290). Digitalized pictures were visualized on a high-resolution color display. The true color image analysis software package (Leica Q Win standard, digital camera CH-9435 DFC 290, Germany) was used for the quantification of the images and data collection.

Immunostaining of NKCs was evaluated by counting CD56-positive cells in five randomly selected fields/slide at 400× magnifications. The microscopic field area was of 786.432 μ².

The IOD [11, 12] of VEGF-A expression was calculated (Figure 1).

![Figure 1 – Screen-shot of measurement of integrated optical density of VEGF-expression using ImageJ software.](image-url)
to a computer for image analysis. The IOD evaluation was done using the image analysis software, ImageJ 2.0.0-beta4 v (National Institutes of Health, USA). Each image was first converted into an 8-bit grayscale profile. Five regions were selected using the ‘selecting tool’ available within the software providing: (a) faint or equivocal immunoreaction was ignored. Tumors were considered positive when they showed moderate or strong staining for VEGF [13], (b) involving different endometrial layers to minimize the effects of varied endometrial epithelium/stromal/gland ratios among the different subjects [14]. IOD was considered for measurement, then the mean IOD was calculated for each slide and the results were exported into Excel sheet.

The quantification of microvasculature density (MVD) – the microvascular profile was determined by CD34 staining as described by Weidner [15]. The entire endometrial section was scanned at low power (×100) to identify the “hot spots”, which represent the areas of highest neo-vascularization. The individual microvessels were later counted under high power (×400) to obtain a vessel count in a defined area. The average vessel count in the five “hot spots” was calculated as the MVD. The results were calculated as endometrial MVD/mm² (vessels/mm²).

Histological and immunohistochemical results

HE-stained sections of normal endometrium revealed normal shaped, regularly arranged uterine glands lined with low simple columnar epithelium. The glands were interrupted by large areas of highly vascular and cellular connective tissue stroma (Figure 2A). The normal proliferative endometrium exhibited proliferating and regularly arranged endometrial glands that appeared normal in shape and outlines and were separated by large amount of connective tissue stroma (Figure 2B). Specimens of typical simple EH exhibited mildly crowded glands lined with columnar epithelium with basal oval nuclei with preserved endometrial stroma (Figure 2C). Specimens of typical complex EH exhibited crowded, disorganized, angulated glands with luminal outpunching that were lined with high columnar epithelium with basal oval nuclei. The endometrial stroma was extremely reduced (Figure 2D). Specimens of atypical EH exhibited extremely crowded and disorganized glands separated by markedly reduced highly vascular endometrial stroma (Figure 2E). The glands were lined with eosinophilic cells with marked nuclear atypia characterized by increased nuclear roundness, clearing of nuclear chromatin with prominent nucleoli, clumping of chromatin and mitotic figures. The stromal blood vessels were extremely congested (Figure 2F).

Statistical analysis

Statistical analysis was performed using the IBM SPSS (version 20) statistical package. Comparisons between groups were assessed using the Kruskal–Wallis or one-way analysis of variance (ANOVA) followed by Fisher’s LSD multiple comparison test when necessary. Correlations among all variables were plotted and corresponding Pearson’s correlation coefficients (r) were reported. Statistical significance was set at p<0.05 for the comparison of means and at p=0.01 for the correlation analysis. The statistical analyses used are described in table footnotes.

Results

Clinical criteria of the study groups

Table 2 presents the comparison of the clinical characteristics between the case and the control groups. Complex endometrial hyperplasia (EH) with atypia was significantly associated with menopause and high body mass index (BMI) compared with the other EH groups (p<0.001 and p<0.001, respectively). The most common clinical presentation for women with EH was abnormal uterine bleeding.
NKC (CD56+) immunoexpression

In the normal endometrium, only a few NKC (CD56+) were detected in the endometrial stroma (Figure 3A), and in cases of simple and complex EH without atypia, NKC (CD56+) were seen only rarely scattered around the glands (Figure 3, B and C). However, a marked increase in the number of NKC (CD56+) was detected in cases with atypical EH, and the NKC were distributed perivascularly and close to the glands (Figure 3D).

Statistical data revealed fewer NKC (CD56+) in simple (2.0±0.22; p=0.06) and complex (2.4±0.22; p=0.43) EH without atypia compared with the normal endometrium (2.7±0.26), but the differences between the groups were not statistically significant. However, there was a marked significant increase in the number of NKC in cases with atypical EH (9.0±0.40) compared with the other groups (p<0.001) (Table 3; Figure 4).
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Figure 3 (continued) – Photomicrographs of human endometrial specimens illustrating the following: (C) Typical complex hyperplasia with few CD56+ NKCs (thin arrows) seen in the stroma, close to the glands (G) and close to the blood vessels (V); (D) Atypical hyperplasia with a marked increase in the number of CD56+ NKCs (arrows) present, mainly close to the blood vessels (V) and glands (G). (Anti-CD56, ×400).

Table 3 – Number of NKCs (CD56+), integrated optical density (IOD) of VEGF-A expression and microvessel density (MVD) in ~1 mm field of view of endometrial specimens from the studied groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal non-hyperplastic endometrium (n=25)</th>
<th>Simple EH without atypia (n=30)</th>
<th>Complex EH without atypia (n=25)</th>
<th>Complex EH with atypia (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of NKCs (CD56+)</td>
<td>2.7±0.26</td>
<td>2.0±0.22</td>
<td>2.4±0.22</td>
<td>9.0±0.40</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IOD of VEGF-A</td>
<td>3 166 006.7±404 346.8</td>
<td>7 901 476.4±1 584 942.6</td>
<td>12 288 976.2±1 584 942.6</td>
<td>17 493 616.1±2 484 822</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MVD</td>
<td>5.4±0.18</td>
<td>6.9±0.38</td>
<td>8.3±0.40</td>
<td>10.32±0.47</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Data is represented as mean±standard error. EH: Endometrial hyperplasia; NKCs: Natural killer cells; IOD: Integrated optical density; MVD: Microvascular density. *Significant p<0.05 for F-test.

Figure 4 – Histogram represents the number of NKCs (CD56+), integrated optical density of VEGF-A expression and microvessels density in a ~1 mm field of view of endometrial specimens from the studied groups.

VEGF-A immunoexpression

VEGF-A was present in all samples of normal and hyperplastic endometrium. VEGF-A was expressed in the cytoplasm of normal endometrial cells, hyperplastic cells, endothelial cells and stroma. Positive VEGF-A immunoreactions exhibited a coloration of variable intensity inside the cytoplasm of endometrial cells and focal intensity in the stromal cells. Inside the proliferative normal endometrium, there were negative or weakly positive VEGF immunoreactions (Figure 5A). In the simple and complex typical EH, relative to normal endometrium, VEGF-A immunoreactivity was elevated, particularly in the apical parts of the glands (Figure 5, B and C). The greatest VEGF-A immunoreactivity was detected in the whole glandular epithelium of atypical EH (Figure 5D).

Statistical data revealed increased IOD of VEGF-A with progression from the proliferative normal endometrium through the three groups of EH and the differences within the groups were statistically significant (Table 3; Figure 4).
The levels of significance \( (p) \) between the different groups were 0.019 between the normal and simple EH, <0.001 between the normal and complex EH without atypia, <0.001 between the normal and complex EH with atypia, 0.03 between simple EH and complex EH without atypia, <0.001 between simple EH and complex EH with atypia and 0.014 between complex EH without atypia and complex EH with atypia.

**CD34 immunoexpression and MVD**

Blood vessels were demarcated by CD34 expression and were scattered throughout the stroma and close to the endometrial glands in the normal endometrium (Figure 6A), typical simple EH (Figure 6B), typical complex EH (Figure 6C) and atypical complex EH (Figure 6D).

MVD increased significantly with progression from the proliferative normal endometrium through the three groups of EH \( (p<0.001) \). Additionally, the newly formed vessels tended to accumulated in the areas where atypia was observed.

Cases with normal endometrium exhibited a mean IOD of VEGF-A = 3166067 and a mean MVD value of 5.4±0.18, whereas cases with simple EH without atypia exhibited a mean IOD of VEGF-A = 7901476.4 and a significantly higher mean MVD \( (6.9±0.38; p=0.004) \). Cases with complex EH without atypia exhibited a mean IOD of VEGF-A = 12288976.2 and a significantly higher mean MVD \( (8.3±0.4; p<0.001) \). Cases of atypical EH exhibited a mean IOD of VEGF-A = 17493616.1 and the highest mean MVD \( (10.32±0.47; p<0.001) \) compared with the other groups (Table 3). Pearson’s correlation revealed a significant correlation between the MVD and the IOD of VEGF-A in all studied cases \( (r=0.332; p=0.001) \) (Figure 7).

Additionally, significant correlations were documented between the density of NKCs (CD56+) and the IOD of VEGF-A \( (r=0.362; p<0.001) \) as well as the endometrial MVD \( (r=0.473; p<0.001) \) in all studied cases (Figure 7).

![Figure 5 – Photomicrographs of human endometrial specimens illustrating the following: (A) Normal endometrium with glandular epithelium (arrows) exhibiting faint VEGF-immunoexpression in some cells of the glands (G); (B) Typical simple hyperplasia with mild VEGF-immunoreactions but negative stroma (S); (C) Typical complex hyperplasia with moderate VEGF immunoreactiviy mainly in the apical part of the glandular epithelium (arrow heads) but negative stroma (S); (D) Atypical hyperplasia with strong VEGF immunoreactivity in the entire glandular epithelium (arrow heads). (Anti-VEGF, ×400).](image-url)
Discussion

Atypical endometrial hyperplasia has been associated strongly with progression to endometrial carcinoma and the presence of concomitant endometrial carcinoma. Although atypical hyperplasia can be treated successfully with progestins, hysterectomy is recommended for postmenopausal women with cytological atypia because of the high risk of coexistent endometrial carcinoma and progression to cancer [16].

With respect to the risk factors associated with endometrial hyperplasia, our results indicate that menopausal status and high BMI are significantly associated with complex atypical hyperplasia. Similarly, other reports have been confirmed increased body weight as a risk factor for EH and adenocarcinoma [17, 18]. There are several possible mechanisms proposed for elevated body mass in endometrial carcinogenesis. First, a weight-related increase in insulin and insulin-like growth factor-I has been observed for endometrial proliferation [19]. Second, cytokines [20, 21] produced from fat tissue and transcription factors [22] related to cellular lipid metabolism and tumorigenesis have also been identified.

The evaluation of general immune mechanisms in various benign pathologies of the endometrium helps to determine effective approaches to differential diagnosis.
and treatment strategies. NKCds (CD56+), are observed occasionally scattered in the endometrial stroma, although these cells exhibit several markers indicating different behaviors throughout the endometrium [23]. These data were confirmed by the present study. There were approximately 2.7 ± 0.26 cells NKCds (CD56+) in ~1 mm in normal non-hyperplastic endometrium. This is the first investigation regarding the density of uterine NKCds in this patient population, and our values were less than those of other reports in different nations, e.g., China [24], Japan [25] and the UK [26]. These differences may be the result of ethnic or racial variance in endometrial immunological profiling [25].

The investigations of the density and distribution of NKCds (CD56+) in endometrial hyperplasia have been limited, and previous reports have primarily focused on the analysis of NKCds in endometrial carcinomas. We found that the NKC count varied both in non-neoplastic and neoplastic endometrial tissue. Compared with non-hyperplastic endometrium, fewer NKCds were observed in cases of hyperplasia without atypia (non-neoplastic lesion), and significantly more NKCds were observed in cases with atypia (pre-neoplastic lesion).

The finding of lower NKCds density in non-neoplastic endometrium relative to non-hyperplastic endometrium is similar to what has been reported in patients with endometriosis [27]. Endometrial NKCds contain cytoplasmic granules for the release of cytotoxic molecules, such as perforin, granzyme and granulysin, in response to cells that lack or are deficient in human leukocyte antigen class I expression [28]. The low endometrial NKCds density may allow the survival of residual endometrial cells and potentially lead to in situ growth. By contrast, Lysenko et al. [29] reported increased numbers of NKCds in patients with adenomatous and glandular endometrial hyperplasia compared with control patients with dysfunctional uterine bleeding without hyperplasia. No additional studies have clearly explored this potential relationship among different histological types of EHA.

Elevated NKCds in patients with complex hyperplasia associated with atypia indicates preserved capacity to mobilize natural immunity and can be evidence of involvement of immune mechanisms in the pathological process in precancerous lesions. The opposite relationship (decrease levels of lymphocytes and increased numbers of NKCds) were reported previously by Lysenko et al. [29] in patients with adenomatous and glandular endometrial hyperplasia, characterizing such patients as immunocompromised. Imbalance in this system can lead to defects in immunocompetent cells with possible consequent impairment of mechanisms of antitumor immune control, which provides conditions for the reproduction of the tumor clone. Jewett & Tseng [30] stated that a majority of NKCds observed in patients exhibit modified phenotypes to support the differentiation of the cells and may therefore be ineffective in eliminating the cancer stem cells. A number of studies have been published on the role of immunologic factors in the pathogenesis of endometrial lesions, associating lesion presence with the presence of various cytokines and with various types of inflammatory responses [31]. The balance between immunity and tolerance is important to maintain immune homeostasis, thus justifying the many mechanisms involved in keeping the immune response under control, including the activity of the NKCds and T-regulatory cells [32].

In the present study, we examined the association between angiogenesis and the histological type of the EH. Angiogenesis was quantified by anti-CD34 staining and expressed as MVD. MVD increased with progression from the proliferative normal endometrium through the three stages of EH, and the differences in MVD counts among the different groups were statistically significant. Thus, as the cytological atypia spreads, the number of new vessels increases. Additionally, the newly formed vessels were concentrated in the areas with atypia. These findings are consistent with many old and recent reports [33–35].

The transition from complex hyperplasia with atypia to endometrial cancer was reported to occur at a rate of 10–26% [34], demonstrating the importance of the study of MVD in complex hyperplasia and the possible contributing factors to prevent disease progression. Recent experiments have begun to yield direct evidence that tumor growth is angiogenesis-dependent [5, 6]. Tumor growth is limited to a few millimeters in greatest dimension before neovascularization but is rapid and nearly exponential after neovascularization. Such neovascularization may be stimulated by factors released from the tumor cells, tumor-associated inflammatory cells, and/or from the extracellular matrix.

In addition, the current study demonstrated that the angiogenic factor VEGF-A was expressed in EH, and the mean IOD of VEGF-A expression was significantly higher in EH than in normal proliferative endometrium. Additionally, we observed a significant correlation between the MVD and the IOD of VEGF in all studied cases. Many studies have reported increased production of VEGF-A in EH and carcinoma relative to normal endometrium [35, 36], although one published report failed to detect VEGF-A mRNA in benign specimens [37]. VEGF-A expression is known to contribute to angiogenesis in the transition towards carcinoma [35, 36].

We also detected significant correlations between the density of NKCds and the IOD of VEGF-A as well as to MVD. Another recently hypothesized a role for VEGF and certain cytokines, such as PGE2 and interleukin-6, in inducing immunosuppression in the tumor microenvironment. This relationship could be related to the close correlation between angiogenic factors and the antitumor effect of NKCds as explained by Jewett et al. [38] and Yaguchi et al. [39]. These authors reported that cancer-associated fibroblasts, which are potential tumor promoters, have the ability to secrete VEGF and that these fibroblasts as well as other tumor promoters are significantly more susceptible to NKC-mediated cytotoxicity.

This immunosuppressive role of VEGF may be supported by the finding that neutralizing antibodies to VEGF can partially reverse the inhibitory effects of tumor cell supernatants on dendritic cell maturation and function. Additionally, increased numbers of immature dendritic cells were observed in the peripheral blood of cancer patients with elevated levels of circulating VEGF [30].

Therefore, VEGF may contribute to tumor development by stimulating both angiogenesis and immunosuppression, thereby promoting cancer cell proliferation. However,
there are contradictory results regarding the prognostic relevance of VEGF and its receptors in the evolution of endometrial carcinoma.

**Conclusions**

The present study demonstrated that uterine CD56+ NKC count increased in patients with complex atypical endometrial hyperplasia relative to hyperplastic endometrium without atypia and non-hyperplastic groups. IOD of VEGF expression and endometrial MVD were significantly increased with the histological progression of hyperplasia. NKC's were significantly correlated with IOD of VEGF and endometrial MVD. These results suggest that increased NKC's count and altered endometrial VEGF expression and MVD are related to the histological changes of the endometrium. Furthermore, our results suggest a distinct immunological mechanism underlying endometrial hyperplasia in the context of NKC infiltration and VEGF production.

**Conflict of interests**

The authors declare that they have no conflict of interests.

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