RESEARCH ARTICLE

Simple Immunocytochemistry from Menstrual Blood in Diagnosis of Endometriosis

Tita H Madjid1, Bethy S Hernowo2

ABSTRACT

Introduction: The menstrual reflux containing viable endometrial cells precede the development of endometriosis lesion. Based on that, we can assume that we can detect the presence of endometriosis by utilizing the endometrial cells in the menstrual blood sloughing. The purpose of this study was to prove that simple immunocytochemistry technique on a sample of menstrual blood may show endometrial cells.

Materials and methods: A case–control study involving 60 women was performed, from February 2007 to February 2008. Screening for suspected endometriosis was performed by history taking, physical diagnosis and additional examination. Diagnostic laparoscopy or laparotomy and biopsy were performed afterward. Endometriosis histopathologically defined as the presence of epithelial cells of endometrial glands and stroma in the tissue being examined. Menstrual blood sampling is performed by taking 20 drops of blood into 20 mL preservative solution. In this study, the cells/tissues staining by immunohistochemistry using diaminobenzene (DAB), and for comparison hematoxylin–eosin staining (HE) was used so that the stromal cells which express caspase-3, caspase-9 and MMP-9 are stained brown with a blue background.

Results: The endometrial cells were successfully isolated using a preservative solution, and all samples from endometriosis subjects could be analyzed for the expression of caspase-3, caspase-9, and MMP-9.

Discussion: The results of this study lead to a conclusion that immunocytochemistry analysis of the menstrual blood endometrial cells can be applied as a noninvasive method for establishing the diagnosis of endometriosis in daily practice.

Keywords: Caspase-3, Caspase-9, Endometriosis, Menstrual blood, Noninvasive.

Journal of South Asian Federation of Obstetrics and Gynaecology (2019): 10.5005/jp-journals-10006-1711

INTRODUCTION

Endometriosis is a progressive gynecological disease, which is characterized by the presence of tissue resembling endometrial glands and stroma in the pelvic peritoneum, and other extraterine tissue. Endometriosis can stimulate chronic inflammatory reaction and endometriosis lesions will proliferate and implant into the surrounding tissues, causing fibrosis, adhesions and organ distortion.1–3

Epidemiologically endometriosis is often experienced by women of reproductive age, of all ethnic and social groups, suffering from chronic pelvic pain, infertility, and disorders of both the intra-abdominal and intrapelvic organs. Internal organ disorders occur because of adhesions, suppression, and obstruction of the gastrointestinal tract, urinary tract and reproductive organs. This disease shows a high recurrence (75%) after treatment is discontinued, it becomes progressive, interferes with daily activities, lowers productivity, causing heavy stress, which ends up with decreased quality of life of the sufferers.1–3

The prevalence of the disease is not precisely known due to constraint of having definite diagnosis of endometriosis where surgical procedure is required and be followed by histopathological verification. The estimated prevalence of endometriosis around the world is 10%, which means that approximately 70 million women worldwide suffer from endometriosis. The disease was first suspected more often in women of reproductive age (25–29 years old), although generally confirmed diagnosis is often too late because women came after infertility complaints. Most endometriosis (3–4.1%) was found by chance when laparoscopy for sterilization in women without complaints and symptoms and higher (around 20%, range 2–78%) during laparoscopy in women with infertility complaints and even higher (24%, range 4–78%) in women with pelvic pain.1–3

On the other hand, the incidence of endometriosis increases from year to year. In the developed countries, endometriosis is a health problem that is quite prominent. In the United States, the disease is found in more than 7 million women, including teenagers. Furthermore, it turns out that endometriosis is the third order of gynecological diseases are often an indication for major surgery (laparotomy) with or without hysterectomy, which takes care for a long time and spends a high cost. The annual fee for the management of this disease in 1995 is 2–6 million US dollars and continued to increase to reach 22 million US dollars a year in 2002.2 Thus it appears that endometriosis is a disease with many problems, ranging from etiopathogenesis (allegedly multifactorial), clinical manifestations, to how diagnostic and therapeutic invasive and...
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Table 1: Detection rate of caspase-3, caspase-9, and MMP-9 in immunocytochemistry of menstrual blood sample of women with and without endometriosis

<table>
<thead>
<tr>
<th></th>
<th>Endometriosis</th>
<th>Without endometriosis</th>
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<tbody>
<tr>
<td></td>
<td>Caspase-3</td>
<td>Caspase-9</td>
</tr>
<tr>
<td>Unreadable</td>
<td>n</td>
<td>%</td>
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<td>Readable</td>
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Endometriosis is a condition where cells from the endometrium (the lining of the uterus) grow in areas of the body other than the uterus. These cells can cause pain and infertility. Diagnosis can be challenging, as symptoms can vary and the disease can be difficult to detect early. Immunocytochemistry involves staining and examining cells under a microscope to detect specific proteins or antigens. In this study, caspase-3 and caspase-9 were used to identify abnormal apoptosis (cell death) in endometrial cells, and MMP-9 was used to identify metalloproteinase activity, which plays a role in the degradation of extracellular matrix tissue.

Caspase-3 and caspase-9 are enzymes involved in the process of apoptosis. When a cell is triggered to undergo apoptosis, caspase-3 and caspase-9 are activated, leading to the cell's programmed destruction. MMP-9 is a type of metalloproteinase that helps degrade the extracellular matrix, which is important in the implantation of endometrial cells.

The table above shows the detection rates of caspase-3, caspase-9, and MMP-9 in the menstrual blood samples of women with and without endometriosis. The results indicate that there is a higher detection rate of caspase-3, caspase-9, and MMP-9 in women with endometriosis compared to those without.

In conclusion, immunocytochemistry can be a useful tool in the diagnosis of endometriosis, particularly when combined with other diagnostic methods such as ultrasonography, laparoscopy, and histopathology. Further research is needed to validate these findings and explore the potential of immunocytochemistry as a diagnostic tool for endometriosis.
the gene. This variation is different in each ethnic.\textsuperscript{12,13,15} The MMP-9 gene polymorphism in the promoter region are involved in the transcription process.\textsuperscript{15–17}

Given that these proteins play an important role in the onset of disease, it is thought that the analysis of caspase-3, caspase-9, and MMP-9 in menstrual blood can be used as markers to diagnose endometriosis. Until now generally, immunocytochemistry technique is still difficult, especially for the isolation of endometrial cells in menstrual blood.\textsuperscript{16} If the endometrial cells in menstrual blood can be isolated and abnormal protein can be detected by immunocytochemistry technique then this method can clarify the pathogenesis of the disease and are also able to diagnose the disease is noninvasive. It is expected to be developed another option diagnosis means that noninvasive. This method can connect the clinical manifestations of endometriosis with a view caspase-3, caspase-9, and MMP-9 in menstrual blood. These findings on the future thought may provide opportunities to develop ways of early diagnosis, management, and prevention of disease in addition to clarify the pathogenesis.

**Materials and Methods**

**Subjects**

The subjects were female patients suspected of suffering from endometriosis who came to the Polyclinic-Fertility Reproductive Endocrinology, Gynecology Clinic FKUP/RSHS, and the hospital network of RSHS. All patients with suspected endometriosis who came went to the Polyclinic of Fertility Reproductive Endocrinology, Gynecology Clinic FKUP/RSHS and hospital networks RSHS much as 150–200 patients for a period of one year. Women with external endometriosis, endometriomas been diagnosed with endometriosis or externally by histopathology results. Controls were women with histopathological results not endometriosis. Exclusion criteria were pregnant women. Being in the treatment of hormone, for at least three months, unless GnRH after nine months from the last administration. Another gynecologic disease, such as infection, malignancy, and other serious illnesses. Suffering from other diseases that complicate research, like other systemic diseases. The number of study participants who collected is sixty in total.

**Clinical Examinations**

Anamnesis, examination, and physical ultrasonography performed by researchers in an attempt to make a diagnosis. In the surgery is performed classification, then taken of the sample tissue. In patients who had surgery, surgery of data collection to determine the degree of the lesion.

**Sample Processing Procedures of Menstruation Blood**

Menstrual blood sampling is performed by dripping 20 drops of blood into 20 mL preservative.

**Immunocytochemistry**

Endometriosis by histopathologic criteria is the presence of epithelial cells of endometrial glands and stroma in the endometrial tissue examined. In this study, staining cells/tissues in immunohistochemistry using dianminobenzene (DAB), and the comparison is used hematoxylin–eosin staining (HE) so that the stromal cells which express MMP-9 and caspase-3 and caspase-9 will be stained brown with a blue background.

**Data Analysis**

The data analysis research conducted descriptive and analytic. Age grouping is intended to eliminate confounding incidence, and sort out cases that occur during adolescence (under 20 years), young adults (20–24 years), the peak age first diagnosed (25–35), and in perimenopause. The descriptive calculation is made by calculating the amount of the number and percentage, whereas analytical calculations were made with the statistical test adapted to the hypothesis to be tested.

**Results**

The study lasted for a year (February 2007–February 2008) in Reproductive Endocrinology Clinic-Fertility and Gynecology FKUP/RSHS and Hospital Network RSHS. Through history, physical examination and other investigations carried out screening of patients with endometriosis, then performed a diagnostic laparoscopy or laparotomy, which ended with a biopsy of the lesion. Biomolecular examination caspase-3, caspase-9, and MMP-9. Based on the results of microscopic histopathology as ascertainment, assessed the relationship of clinical manifestations of endometriosis cases and not endometriosis with menstrual blood biomolecular markers. Found 63 (42.28%) cases of endometriosis, while 86 (57.72%) classified as not endometriosis.

Each immunocytochemistry result is then interpreted according to the color criteria which shows the strength and weakness of the protein by endometrial cells (histoscore), as follows:

- Intensity+: light brown in color
- Intensity+: brown
- Intensity+++: dark brown in color

We found the immunocytochemistry of caspase-3 obtained from first day of menstrual blood of women with endometriosis showed higher expression compared to that obtained from non-endometriosis women (Fig. 1).

We tried to observe the expression of caspase-3 obtained from the third day of menstrual blood, and we found fewer cells stained with caspase-3. Yet comparison between women with endometriosis still showed higher expression of caspase-3 compared to that of nonendometriosis women (Fig. 2).

In our study, we also showed the expression ratio caspase-3/ caspase-9 was higher in patients with endometriosis compared to that of nonendometriosis (Fig. 3).

**Discussion**

Physiologically apoptosis was detected in endometrial glandular epithelium which is at the end of the secretion phase and menstrual phase, while little apoptosis was detected during the proliferative phase or initial phase of secretion. Eutopic endometrium of endometriosis patients changes like ectopic tissue. The changes are not found in eutopic endometrium of women without endometriosis. This fact has led to the view that there is a primary defect in endometriosis in eutopic endometrium. The elements of cells and tissues derived from the change eutopic endometrium and overflow into the peritoneal cavity allegedly high potential for implantation and grow on the surface of the peritoneum, developing into endometriosis. The reduced apoptosis in cells suspected endometriosis is an important factor in the development of this disease.\textsuperscript{6,12,13} In this study, patients with endometrial cells of endometriosis based disorders endometrial response to progesterone expected found intact and alive because...
Results of this study confirm what previous researchers claim that endometrial cells can be found either in the network endometriosis (ectopic endometrium), an abundance of menstrual blood and peritoneal fluid.\textsuperscript{13,19} It has been thought that the analysis

of impaired apoptosis, so that they can live longer. As a regulator of apoptotic caspase enzymes in endometriosis experiencing irregularities, thus weakening apoptosis and caspase shown decreases in endometrial cells from menstrual blood.

Figs 1A and B: (A) Immunocytochemistry of endometrial cells from the first day of menstrual blood from women with endometriosis; (B) Without endometriosis

Figs 2A and B: (A) Immunocytochemistry of endometrial cells from the third day of menstrual blood from women with endometriosis; (B) Without endometriosis

Figs 3A and B: The expression of caspase-3/caspase-9 in endometrial cells from menstrual blood; (A) Endometrial cells from menstrual blood without expressions of caspase-3/caspase-9 (magnification 200×); (B) Endometrial cells from menstrual blood with expressions of caspase-3/caspase-9 (magnification 400×)
of cells through menstrual blood cannot be done because it is already necrosis and only a few cells are found so a lot of research endometriosis use of scrapings/endometrial biopsy or make menstrual blood culture.

Results showed that there was no difference in appearance rating protein caspase-3, caspase-9 and MMP-9 in the endometriosis group than non-endometriosis. The preparation cannot be analyzed is the preparation that does not contain endometrial stromal cells, while other cells there looked good (it can be analyzed) although the amount is too little. This condition is thought that menstrual blood is taken after the expiration of the first days of menstruation because it is important ascertained menstrual blood storage time is right, namely the first or second day of menstruation. Thus, this study managed to alienate menstrual blood and endometrial cells also displayed satisfactorily managed and can be analyzed properly, so the chances to diagnose and assess prognosis noninvasive endometriosis can be further developed.

Results showed negative expression of caspase-3 and caspase-9 tend to be greater in patients with endometriosis compared to normal women. The differences of each examination for the two groups were not significant. Odds ratios for caspase-3 is 1.43. This means that women with endometriosis have a display alleged caspase-3 were negative, and 1.43 times likely to have endometriosis compared to without endometriosis. Similarly, when the caspase-9 negative views on women with suspected endometriosis, then the chances of suffering from endometriosis is 5.46 times compared to women without endometriosis.

MMP-9 is a protein that plays a role in the destruction of the extracellular matrix, due to the properties of these cells were able to implant once attached to the peritoneum. Expression of MMP-9 (+3) and the expression of MMP-9 (+2) in both groups the difference was not significant. Have believed that strong apoptosis disorders prevalent in endometriosis. In this study, the percentage of expression of MMP-9 (+2) more precisely, while all cases of endometriosis showed expression of caspase (+3) and (+2). Ten cases (29%) endometriosis that normal visual assessment showed the expression of MMP-9 (+2). An odd ratio value of 1.72 states that in this study patients with endometriosis to display its MMP-9 in endometrial cells (+2) and (+3), likely to have endometriosis by 1.72 times higher than normal women. As the results of the immunocytochemistry for caspase-3 and caspase-9, increased expression of MMP-9 in endometriosis tend to be higher than non-endometriosis (Fig. 3).

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seen on the first day and then the next day is becoming rarer but still visible on day 2 and 3. For patients whose decay endometrium much going on day 2, then the blood sample of 0.5 cc (10 drops) cannot be collected on the first day, and the exposure of endometrial cells can still be seen on the 3rd and 4th with clumps of cells are more dispersed. Depending on the day to how the decay of the endometrium is greatest (day 1 or 2), the endometrial cells are still exposed either to be read to day to 3 for which decays largest on the 1st or 4th day of menstruation in menstrual decays highest on the day 2 menstruation.

**Conclusion**

The results of this study lead to a conclusion that immunocytochemistry of the menstrual blood endometrial cells can be applied as a noninvasive method for establishing the diagnosis of endometriosis in daily practice. The selection of protein markers that are more specific and sensitive still needed.

**References**