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RESEARCH

Correlation Expression Immunocytochemistry Vascular Endothelial Growth Factor A (VEGF A) With Protein Gene Product 9.5 (PGP 9.5) of Menstrual Blood On Pathophysiology Endometriosis

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Abstract

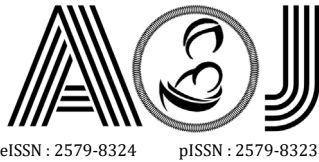
There are changes of eutopic endometrium molecular fenotype in endometriosis such as changes in gene expression, steroid hormone response, increase of inflammation marker and cellular adhesion molecule, decrease of apoptotic index and decidualization capacity, increase of oxidative stress marker, increase activity of angiogenesis and neurogenesis. This study was conducted to analyze the differences in the expression of angiogenic factors (VEGF A) and neurogenesis factors (PGP 9.5) eutopic endometrium of menstrual bleeding among patients with endometriosis and non endometriosis and then the correlation of the two factors. This study is a cross sectional that examines the relationship between the incidence of endometriosis with risk factors such as VEGF expression and PGP 9.5 in menstrual blood. There were significant differences in the expression of VEGF A eutopic endometrium menstrual blood between endometriosis and non endometriosis group ($p=0.002$). There are significant differences expression of VEGF A and PGP 9.5 eutopic endometrial of menstrual blood between endometriosis and non endometriosis groups. There is a positive correlation between the expression of VEGF A with PGP 9.5 eutopic endometrial of menstrual blood on endometriosis patients.

Keywords: eutopic endometrium, menstrual blood, VEGF A expression, PGP 9.5 expression, immunocytochemistry

INTRODUCTION

There are changes in the molecular phenotype eutopic endometrium in the form of endometriosis changes in gene expression, response to hormones steroids, increased inflammatory markers and molecules cellular adhesions, decreased apoptotic index and decidualization capacity, increased stress markers oxidative, increased angiogenesis activity and neurogenesis. This molecular change is not affected by surgery on the cyst endometriosis, this is one of the causes of high recurrence rate as high as 75% post-surgery within 2 years.^{1,3}

This research uses a sample endometrial biopsy that proves its presence a significant increase in pro-angiogenic factors potent (VEGF A) in the late and early secretory phases menstruation sufferers of endometriosis. Existence reflux of eutopic cavity endometrial



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fragments peritoneal during menstruation carries angiogenic factors (VEGF A) is thought to play a role against the development of peritoneal endometriosis lesions especially VEGF-rich and red lesions is an early stage of implantation. ⁴⁻⁸

Another study used a biopsy sample the endometrium also shows an increase significant density of nerve fibers in the lining functional and eutopic basal endometrium endometriosis. Sensory nerve fibers were also found small in diameter and non-myelin in the layer functional eutopic endometrium of sufferers endometriosis with a sensitivity of 95% and specificity 100%. Increased density of these nerve fibers contributes to the pain that is felt by people with endometriosis. ⁹⁻¹²

Formation of nerve fibers usually following neovascular formation. Nerve fibers found in the stromal lesions endometriosis located adjacent to immature blood vessels resulting from neoangiogenesis. Nerve Growth Factor (NGF) activities increases in the eutopic endometrium play a role as an influencing growth factor in the process of neurogenesis and angiogenesis. The discovery of the VEGR-2 receptor on the progenitor neuron cells show a lead relationship mutual influence (reciprocal) between vascular endothelium with neuron cells. Antiangiogenesis (cabergoline) not only reduces vessel density blood but also the density of nerve fibers. ^{3,13-16}

METHODS

This study is a cross-sectional study (cross-sectional study) that analyzes the relationship between the incidence of endometriosis with risk factors, namely the expression of VEGF A and PGP 9,5 Eutopic endometrium of menstrual blood. This research was conducted in gynecology and polyclinics endocrinology Dr. Hasan Sadikin Bandung University Medical Faculty Padjadjaran period February to May 2016.

The study population was a patient diagnosed based on endometriosis cyst clinical examination and ultrasound. Research samples are patients who are of reproductive age, regular menstrual cycle last 3 months, no get hormonal therapy in the last 3 months and histopathologic results are following endometriosis. Patients whose histopathological results were not endometriosis put in the control group. Total The sample of this research is 20 people in the group endometriosis and 20 non-endometriosis people, calculated based on the sample formula for the hypothesis comparative non-parametric categorical data no in pairs. Menstrual blood study subjects on the day 1st or 2nd taken using a 3 cc syringe posterior fornix area or OUE. About 10 drops of menstrual blood are put into a preservative solution, shaken and sent to the Pathology laboratory Anatomy to make a slide for examination immunocytological. Menstrual blood obtained fixed with alcohol (methanol) then cells isolated stained immunocytochemically using Diamino Benzen (DAB) and outward comparison using Hematoxylin Eosin (HE) so the stromal cells are expressing VGEF and PGP



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9.5 would brown on blue background. The degree of intensity of the color brown describes degrees of expression of VEGF and PGP 9.5. Assessment immunocytochemical expression of VEGF and PGP 9,5 done qualitatively using a microscope with a magnification of 40-200x with assessment is negative (blue), weakly positive (light brown color), medium positive (brown color) and strongly positive (dark brown color).

The statistical analysis used to assess the differences in VEGF A and PGP 9.5 expression between the endometriosis and non-endometriosis groups was the Mann-Whitney test. Meanwhile, the correlation analysis between VEGF A expression and PGP 9.5 used Spearman correlation (non-parametric) with a confidence degree of 95%. RESULTS The characteristics of the study subjects were distinguished by age and parity. By using the Mann-Whitney test, the p-value was obtained > 0.05, which means that there was no significant difference in the age and parity of the endometriosis and non-endometriosis groups so that it was feasible to be compared as shown in Table 1.

Table 1. Characteristics of Research Subjects

Karakteristik	Kelompok		Nilai <i>p</i> Mann-Whitney
	Endo metriosis n = 20	Non Endo Metriosis n = 20	
Usia (th)			
Mean ± SD	34,25 (7,45)	37,95 (8,22)	0,076
Median	33,0	39,0	
Rentang (min- mak)	23-47	19-48	
Paritas			
Mean ± SD	1,00 (1,03)	1,50 (1,32)	0,26
Median	1,00	1,00	
Rentang (min- mak)	0 – 3	0 - 5	
Uji Shapiro-Wilk (Normalitas Data)			0,042

The data in Table 2 shows that there is a significant difference in VEGF-A expression between the endometriosis and non-endometriosis groups with a value of $p = 0.002$ ($p < 0.05$). This means that VEGF A expression was significantly stronger in menstrual blood in the endometriosis group than in non-endometriosis groups.



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Website:<http://jurnalobgin.fk.unand.ac.id/index.php/JOE>**Table 2.** Differences in Expression of VEGF A

Ekspresi VEGF	Kelompok		Nilai p
	Endometriosis	Non-endometriosis	
Kuat	11(55%)	4 (20%)	0,002
Sedang	8 (40%)	5 (25%)	
Lemah	1 (5%)	11 (55%)	
Total	20 (100%)	20 (100 %)	

There was a significant difference in PGP expression of 9.5 between the endometriosis and non-endometriosis groups with a value of $p = 0.001$ ($p < 0.05$). Nerve fibers are found in 90% of menstrual blood endometriosis. This can be seen from the data in Table 3.

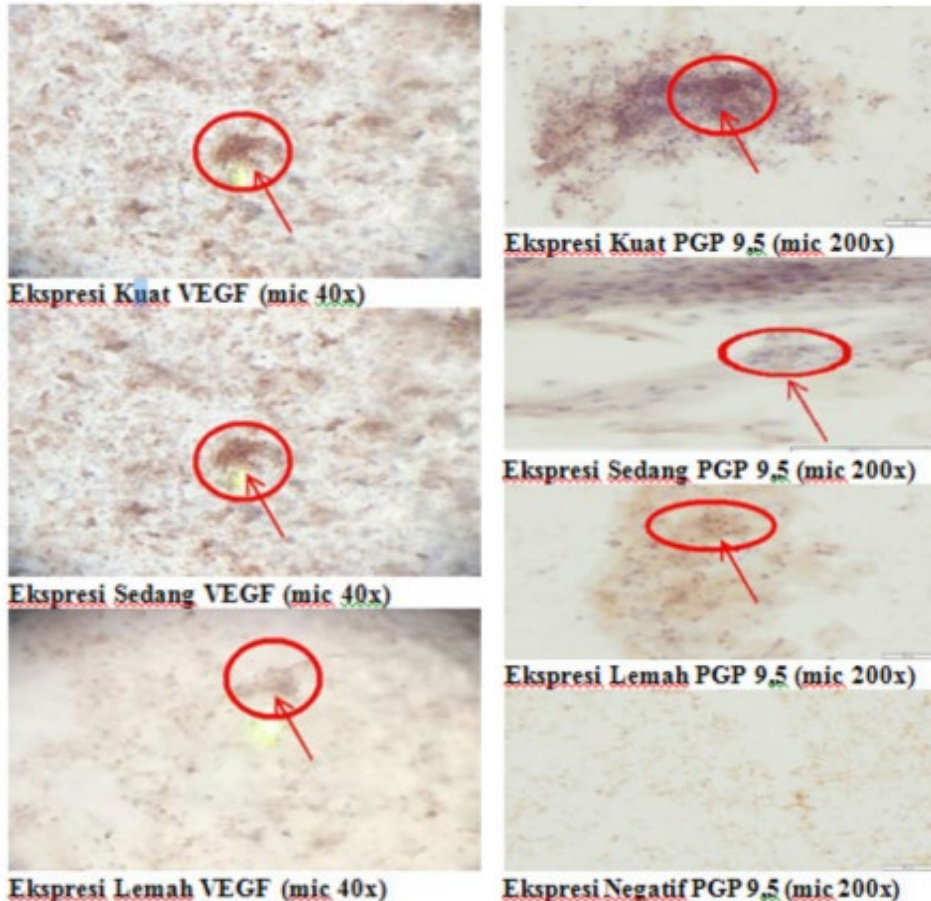
Table 3. Differences in PGP Expressions 9,5

Ekspresi PGP 9.5	Kelompok		Nilai p
	Endo metriosis	Nonendo metriosis	
Negatif	2 (10%)	17 (85%)	0,001
Kuat	3 (15%)	3 (15%)	
Sedang	5 (25%)	0 (0%)	
Lemah	10 (50%)	0 (0%)	
Total	20 (100%)	20(100%)	

The Spearman correlation analysis test between VEGF A expression and PGP 9.5 in the endometriosis group showed a significant ($p < 0.05$) and positive ($r = 0.384$, DK 95%) correlation, seen from the data in Table 4.

Table 4. Correlation of VEGF A Expression with PGP 9.5

Kelompok	Nilai r	Nilai p	Interprestasi
Endometriosis	0,384	0,047	Bermakna / lemah
Non Endo metriosis	0,647	0,001	Bermakna / sedang
Endometriosis + Nonendo metriosis	0,713	0,001	Bermakna / kuat

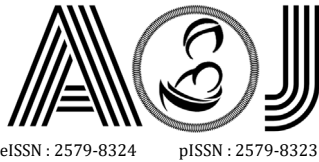
**Immunocytochemical examination results****DISCUSSION****Expression of VEGF A Endometrium Eutopic Endometriosis**

Eutopic endometrium in endometriosis patients is different from non-endometriosis. There are molecular changes, including an increase in angiogenic and neurogenic activity.¹

Garcia, et al. 2007 found a significant increase in VEGF expression in peritoneal fluid, endometriotic implants, and in the endometrioma capsule. There was no correlation between serum VEGF levels and cellular VEGF taken from the endometrial biopsy. 17

Nogueira, et al. 2007 found a significant increase in the VEGF expression of peritoneal fluid in the late and early menstrual secretion phases.

A previous study by Donnez, et al. 1998 also found a significant increase in the expression of VEGF endometrium eutopy glandular epithelium in the final secretion phase in patients with endometriosis.



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Djokovic, 2014 in the literature review states that in endometriosis there is up-regulation of proangiogenic factors so that there is a shift in the balance of antiangiogenic factors.

The results of this study are in line with previous studies, namely that there was a significant increase in the expression of potent proangiogenic factor (VEGF-A) in the ectopic endometrium of menstrual blood in the group of endometriosis sufferers. The difference in this study compared to the previous one was from the sampling technique, namely menstrual blood which was non-invasive, not from a semi-invasive endometrial biopsy.

The increased angiogenic activity of peritoneal fluid and endometriotic lesions has long been demonstrated and widely accepted. The increased angiogenic activity in the eutopic endometrium of patients with endometriosis has attracted the attention of experts because it is suspected that angiogenic factors brought on by reflux to the peritoneal cavity contribute to the formation of red lesions of endometriosis (rich in VEGF) which is the initial phase of implantation of ectopic endometrium in the peritoneal cavity. There is an increase in the potent proangiogenic factor of the eutopic endometrium which follows reflux during menstruation along with other factors causing the endometrial fragments to survive, differentiate, and proliferate at ectopic sites.

This VEGF proangiogenic factor induces a neoangiogenesis process for neovascular formation that supplies nutritional needs and the metabolism of endometrial fragments in the avascular-realistic peritoneal cavity. Increased angiogenic activity of the eutopic endometriosis is the initial stage of neovascular formation before the inflammatory process that invites the arrival of mast cells and macrophages that participate in producing VEGF and other proangiogenic factors. The process of eutopic endometrial angiogenesis, which increases significantly in endometriosis, is the initial part of the development of the lesion.
20,2,19,17

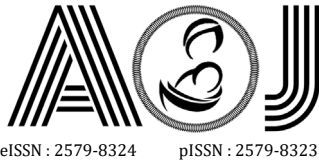
Expression of PGP 9.5 Eutopic Endometriosis

A significant increase in the density of nerve fibers (myelin and non-myelin) is not only found in the stroma of endometriosis lesions but also in the eutopic endometrium, both in the functional and basal layers.^{21,22}

Mechsner, et al. 2007, Yao H, et al. 2010, Arellano, et al. 2010 found an increase in nerve fiber density in peritoneal endometriosis lesions associated with pain intensity.

Tokushige, et al. 2006, Al-Jefout, et al. 2007 used endometrial biopsy samples and found an increase in the density of nerve fibers in the functional and basal layer of endometriosis sufferers. This study found the presence of small, non-myelinated (type C) sensory nerve fibers in the functional layer of the eutopic endometrium of endometriosis sufferers.

The research of Tokushige, et al. 2006 was completed by Bokor, et al. 2009 by continuing to use an endometrial biopsy to examine the entire functional layer of the eutopic



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endometrium immunohistochemically, especially for patients with stage I-II (minimal-mild) endometriosis. The research of Bokor, et al. Succeeded in finding small non-myelinated sensory nerve fibers in the functional layer of the eutopic endometrium with diagnostic values of 95% sensitivity, 100% specificity, 100% PPV, 95% NPV and 97.5% accuracy.

The difference in this study compared to previous studies was that the sampling technique used menstrual blood instead of endometrial biopsy so it was non-invasive. This study was conducted only in patients with endometriosis cysts (stage III-IV) to complement the existing pathophysiology. The results of this study are in line with previous studies, namely the finding of a significant increase in nerve fiber expression in the eutopic endometrium of endometriosis patients. Small sensory nerve fibers non-myelin menstrual blood are found in 90% of patients with endometriosis and 15% of non-endometriosis. The presence of nerve fibers in menstrual blood without endometriosis was caused by confounding factors, namely endometriosis stage I-II which escaped screening so that it was included in the control group. The absence of menstrual pain and the presence of deep insertion lesions that were not visible per laparoscopic were confounding factors for the control group. Future studies need to examine the relationship between increased expression of eutopic endometrial nerve fibers in menstrual blood in patients with endometriosis and pain intensity.

Correlation of VEGF A Expression with PGP 9,5 Eutopic Endometriosis

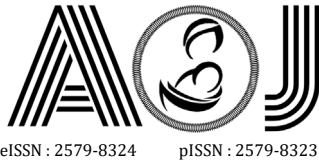
Other studies have found a link between new (immature) blood vessels and the formation of new nerve fibers. Meschner, et al. 2009 found that new nerve fibers in the stroma of endometriosis lesions are located adjacent to immature blood vessels.

Nico, et al. 2008 said that NGF has a neurotrophic effect (sympathetic neurons) and also as an angiogenic molecule that affects endothelial cells and blood vessels. NGF contributes to the maintenance, survival, and function of vascular endothelial cells through autocrine and paracrine mechanisms.

Laura C, et al. 2010 stated that NGF can affect angiogenic activity. In peripheral sensory nerve fibers, NGF stimulates VEGF production as the most potent angiogenic factor in endothelial cell mitogenesis.

Emanuelli, et al. 2003 have stated that NGF stimulates vascular endothelial cell proliferation and has a functional role in neovascularization repair. VEGF A can bind to neuropilin receptors. Angiogenesis and neurogenesis are paracrine regulated by growth factors released by endothelial cells and neurons.²⁵

Jin K, et al. 2002 stated that VEGF is an angiogenic protein with neurotrophic and neuroprotective effects. VEGF promotes endothelial cell proliferation and stimulates the proliferation of neuronal precursors. Zhang, et al also said VEGF directly guided neuron cell progenitors. Neuron progenitor cells also express VEGF receptors.²⁶



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The results of this study are in line with the results of previous studies. In previous studies, the relationship between VEGF expression and nerve fibers was carried out on peritoneal fluid and endometriosis implants. Whereas in this study the relationship between the expression of these two factors was carried out on the eutopic endometrium of menstrual blood. The results of this study showed a significant and positive correlation between VEGF A and PGP 9.5 menstrual blood. The nerve fibers detected by PGP 9.5 of menstrual blood are small non-myelin (type C) sensory nerve fibers that are only found in patients with endometriosis.

The results of this study are also supported by research by Novella, et al. 2012 which found that giving anti-angiogenic (cabergoline) to peritoneal endometriosis would reduce microvascular density and nerve fiber density, thus bringing new hope for pain treatment in endometriosis sufferers.

The results of this study prove the previous theory that there is a reciprocal relationship between angiogenesis and neurogenesis in endometriosis lesions and also in eutopic endometrium.

CONCLUSION

There was a significant difference in the expression of VEGF A and PGP 9,5 eutopic endometrium of menstrual blood between the endometriosis and non-endometriosis groups.

There is a positive correlation between the expression of VEGF A and PGP 9,5 eutopic endometrium of menstrual blood in patients with endometriosis. The interplay between the process of angiogenesis and neurogenesis brings new hope to the development of anti-angiogenic use as a therapeutic modality for pain management in endometriosis sufferers.

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