Nitric oxide (NO) level of the follicular fluid in endometriosis patients

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ORIGINAL ARTICLE:

Nitric oxide (NO) level of the follicular fluid in endometriosis patients

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ABSTRACT

Objectives: To ascertain the nitric oxide (NO) level of the follicular fluid of endometriosis sufferers compared with that of the non-endometriosis patients.
Materials and Methods: Follicular fluid was taken from 64 women, but only 54 continued with further laboratory test. Women with endometriosis had their follicular fluid taken during laparoscopy, while the follicular fluid from non-endometriosis women was taken during tubectomy. Nitric Oxide level was analyzed using the PCR-RFLP method. Data were analyzed using software STATA Intercooled Version 11.
Results: NO level in endometriosis is higher than that in non-endometriosis and has a significant relationship with all the variables (age, parity, dysmenorrhea, dyspareunia, types of endometriosis, and grade of endometriosis).
Conclusion: The NO level in endometriosis is higher than that in non-endometriosis. The distribution of the polymorphism of allele GG is greater than that in non-endometriosis. The existence of polymorphism GG caused a higher eNOS, increasing NO level, causing a decrease in fertility which derived from the degenerative oocyte.

Keywords: nitric oxide; endometriosis; follicular fluid.

ABSTRAK

Tujuan: Untuk menentukan kadar oksida nitrat (NO) dari cairan folikel penderita endometriosis dibandingkan dengan pasien non-endometriosis.
Bahan dan Metode: Cairan folikel diambil dari 64 wanita, tetapi hanya 54 yang dilanjutkan dengan uji laboratorium. Cairan folikel dari penderita endometriosis diambil selama laparoskopi, sedangkan cairan folikel dari penderita non-endometriosis diambil selama tubektomi. Kadar oksida nitrat dianalisis menggunakan metode PCR-RFLP. Data dianalisis menggunakan perangkat lunak STATA Intercooled Versi 11.
Hasil: Kadar NO pada endometriosis lebih tinggi daripada non-endometriosis dan berhubungan signifikan dengan semua variabel (usia, paritas, dismenore, dispareunia, jenis endometriosis, dan derajat endometriosis).
Simpulan: Kadar NO pada endometriosis lebih tinggi daripada non-endometriosis. Distribusi polimorfisme alel GG lebih besar daripada non-endometriosis. Keberadaan polimorfisme GG menyebabkan eNOS lebih tinggi, meningkatkan kadar NO, menyebabkan penurunan kesuburan yang berasal dari oosit degeneratif.

Kata kunci: nitrat oksida; endometriosis; cairan folikel

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INTRODUCTION

In the pathogenesis of endometriosis, the immune system factor is believed to be involved in the progressiveness of endometriosis. The immune system of women with endometriosis will decline, thereby causing the peritoneal fluid in the peritoneal cavity of endometriosis sufferers to short change in the levels of prostanoid, cytokine, growth factor, interleukins, and oxidative stress (OS).[1] The systemic immunoglobulin whereas the immunological disorder of the peritoneum marked by an increase in the T helper cell. The defect in NK cell activity, a decrease in the suppressor cell activity, the proliferation of lymphocytes, decreased binding of the pellucid zone also happened. However, there will be an increase in the cycle of the activation of the macrophage and the presence of non-organ specific antibody, which is characterized by as an immunological abnormality of the peritoneum.[2]

Activation of the macrophage will increase the level and production of nitric oxide (NO) and prostaglandin so that the number of free radicals in the peritoneal fluid of an endometriosis patient also increased.[3] These free radicals will cause oxidative stress in the endometrium. Oxidative stress is associated with the pathophysiology of various diseases because the products of free radicals and lipid peroxidase can destroy the cellular components such as membrane, protein, lipid, and DNA. Oxidative stress along with the granulose apoptosis of cells also affect the quality of oocytes. Endometriosis patients experience increased oxidative stress caused by the excessive production of ROS compared with the defense of antioxidants.[2]

Nitric oxide is known as an intra and extra cellular mediator and has been understood to have the role in the development of a biological process including the physiology of the ovary. During the atresia process, IL-1β presses the apoptosis cell that assumed mediated by NO.[4] In the human luteal culture, the addition of NO will significantly inhibit the production of estradiol and progesterone from the luteal cells, which shows that NO may involve as a regulator of autocrine and steroid arrangement.[5] NO is known to participate in the process of ovulation. Because ovulation causes a general change on inflammation in the ovarian duct of mice, IL-1β is assumed to play a role in ovulation, i.e., to induce eNOS.[6]

Other researchers show that the number of follicles that develops significantly affects the NO con-centration in the follicles, and the IL-1β gives as an additional contribution to the NO generation in the human preovulation follicles.[7] In endometriosis, there is an increase in some macrophages, besides an excessive release of cytokine and other mediators like NO. It knew that NO is a free radical and a bio regulator of apoptosis.[8] On a small level, NO is very important in the function of ovary and implantation, but the vast number of NO and NOS has discovered in the endometriosis of women with endometriosis. The peritoneum of endometriosis patients also shows a high level and activity of NOS. The high level of NO associated with the degree of fertility, and abstract the ovarian tract and sperm duct. This research aims to ascertain the nitric oxide (NO) level of the follicular fluid of endometriosis sufferers compared with that of the non-endometriosis patients.

MATERIALS AND METHODS

This study is cross-sectional with the independent variable of endometriosis types of endometriosis, and grades of endometriosis. The dependent variable is NO, while the external variable is ethnicity, age, dysmenorrhea, BMI, and history of endometriosis in the family. Research on the NO level uses the ELISA method. Data collection did on the endometriosis patients and healthy patients. When performing laparoscopy, the follicular fluid took from the endometriosis patients.

The samples were endometriosis patients that diagnosed according to the ASRM criteria with the non-endometriosis patients who were undergoing tubectomy in the Permata Hati Clinic of Dr. Sardjito Hospital, Yogyakarta. The patients subjected to hysteron-laparoscopy or laparotomy. Samples were taken on the 12th until 15th day using Stor2 aspirating needle, while samples of non-endometriosis took with a syringe. All the endometriosis patients and control who showed NO value involved as respondents. The research conducted from January 30, 2012, to January 30, 2015. The laboratory used was the Biochemistry Laboratory of the UGM Faculty of Medicine to ascertain the NO level using the PCR-RFLP method. Data analysis uses software STATA Intercooled Version 11, using chi-square, t-test, multi linear regression, and logistic multi nominal regression.

RESULTS AND DISCUSSION

The research population was 64 patients, all of whom used as samples or research subjects. Out of the 64 subjects, 54 were able to take part in the laboratory examination, and ten subjects did not continue to participate in the research for various reasons, such as the insufficient samples of ovarian fluid for analysis. 54 patients were selected to participate in this research.

Characteristics of clinical distribution of endometriosis

Table 1. Characteristic of clinical distribution

Variables	Endometriosis		Non-Endometriosis	
	N	%	N	%
Grade				
0 (no-endomet.)	0	0.00	27	100.0
2	3	11.11	-	-
3	11	40.74	-	-
4	13	48.15	-	-
Endometriosis				
Chocolate (-)	11	40.74	-	-
Chocolate (+)	16	59.26	-	-
Genotype				
GG	7	4.7	1	6.7
GT	6	40.0	7	46.7
TT	2	13.3	7	46.7

Table 1 shows the characteristic of the clinical distribution of endometriosis patients. The laparoscopy procedures for ascertaining the grade of endometriosis found dominant at stage 4. For the type of endometriosis, it was higher in patients with negative-chocolate cyst compared to a positive-chocolate cyst. The mean level of nitric oxide level was 7.42 μmol/L (up to $\pm$ 1.84).

The NO level in endometriosis

The correlation between the incidence of endometriosis and NO level presented in Table 2. For examination of nitric oxide in endometriosis and non-endometriosis patients, aspiration of follicular fluid in the ovary was done by laparoscopy. In endometriosis and non-endometriosis patients, the NO level were respectively 8.62 (up to $\pm$ 1.87) and 6.53 (up to $\pm$ 1.32) with P=0.01. This study showed that the NO level in endometriosis patients was significantly different from non-endometriosis.

The differences of mean value, age, parity, dysmenorrhea, history of endometriosis in the family, types of endometriosis, grades of endometriosis, and GG/GT genes form nitric oxide evaluated in Table 3. The mean value of NO for the endometriosis variable in endometriosis and non-endometriosis patients was 8.32 (up to $\pm$ 1.87) and 6.54 (up to $\pm$ 1.32). The median difference of the NO level between endometriosis and non-endometriosis patients was 1.78, P value=0.01 and CI 95%= 0.89-2.66.

For the dysmenorrhea variable, the median value of the NO level in endometriosis and non-endometriosis patients was 7.97 up to $\pm$ 2.09 and 6.88 up to $\pm$ 1.38 with the median difference of 1.09, P value=0.02. The variable of parity, dyspareunia, type of endometriosis chocolate (-), type of endometriosis chocolate (+), endometriosis grade 2, endometriosis grade 3, endometriosis grade 4, GG and GT also show a significant median difference of NO level.

Multiple linear regression tests between endometriosis, types of endometriosis, grades of endometriosis, dysmenorrhea, history of endometriosis in the family, and nitric oxide. Table 4 shows model 1 in the endometriosis variable, coefficient value of 1.98 (CI 95%=0.69-3.26 with p=0.01). In the variable of the history of endometriosis in the family, the coefficient value was 0.48 (CI 95%=1.52-2.49 with p=0.63). Endometriosis patients with the history of endometriosis in the family were likely to have on an increase of 29% in nitric oxide.

Model 2 shows that patients with endometriosis of chocolate cyst (-), the coefficient value was 2.002 (CI 95%=0.30-3.74 with p=0.02) whereas patients with endometriosis of chocolate cyst (+), the coefficient value was 1.95 (CI 95%=0.55-3.36 with p=0.00). In the variable of a history of endometriosis in the family, the coefficient value was 0.72 (CI 95%=-1.31-2.75, P= 0.47). Endometriosis patients with a history of endometriosis in the family were likely to have an increase of 30% in nitric oxide.

Table 2. The Independent t-test of NO level between endometriosis and non-endometriosis

Variable	Endometriosis		Non-Endometriosis		P	Δ	CI 95%
	Mean	SD	*Mean*	SD			
NO	8.32	1.87	6.53	1.32	0.01*	1.78	0.89-2.66

* Significant P=<0.05.

Table 3. The NO mean of level between age, parity, dysmenorrhea, history of endometriosis in the family, types of endometriosis, grades of endometriosis, and GG/GT genes

Variable	Nitric Oxide			*P*	Δ	CI 95%
	N	Mean	SD			
Endometriosis						
	27	8.32	1.87	0.01*	1.78	0.89-2.66
Non-endometriosis	27	6.54	1.32	-	-	-
Age						
≤34 years	35	7.78	1.91	0.05	1.01	-0.01-2.03
≥35 years	19	6.77	1.54	-	-	-
Parity						
0-1	27	8.32	1.87	0.01*	1.78	0.89-2.66
2-6	27	6.53	1.32	-	-	-
Dysmenorrhea						
Yes	27	7.97	2.09	0.02*	1.09	0.12-2.05
No	27	6.88	1.38	-	-	-
Dyspareunia						
Yes	5	9.03	2.14	0.03*	1.77	0.08-3.44
No	49	7.26	1.75	-	-	-
Endometriosis (types)						
Chocolate (-)	11	8.23	2.10	0.01*	1.69	0.51-2.86
Chocolate (+)	16	8.38	1.76	0.01*	1.84	0.8-2.88
Not Endometriosis	27	6.53	1.32	-	-	-
Endometriosis (Grades)						
0	27	6.54	1.32	-	-	-
2	3	9.56	1.69	0.01*	3.02	1.04-5.00
3	11	8.03	1.87	0.01*	1.49	0.32-2.65
4	13	8.28	1.92	0.01*	1.74	0.64-2.83
SNP DNA						
GG	8	8.71	1.87	0.01*	3.01	1.01-5.01
GT	13	7.69	2.54	0.03*	1.98	0.19-3.77
TT (Ref)	9	5.70	0.89	-	-	-

* Significant *P*=<0.05

Model 3 show the endometriosis variable grade 2, coefficient value of 2.69 (CI 95%=0.65-4.73, *P*=0.01). In the endometriosis variable grade 3, the coefficient value was 1.39 (CI 95%=-0.17-2.62 *P*=0.02). In the endometriosis variable grade 4, the coefficient value was 1.46 (CI 95%=0.31-2.62 *P*=0.01). In the variables of dysmenorrhea, history of endometriosis in the family, and interactions of all the variables, the p value obtained were respectively 0.08, 0.70, and -0.01. Patients with endometriosis, after considering dysmenorrhea, history of endometriosis in the family, and interactions of all the variables had an increase of 29% in NO level.

Logistic multinomial regression analysis: Relation of NO considering BMI between grade 2 and non-endometriosis between stage 3 and 4. The relationship of IL-1B, NO, 8-OH-dG considering BMI between Stadium 2 and non-endometriosis between Stadium 3, four are presented in Table 5.

Table 5 shows the results of logistic multinomial regression analysis of the relation of NO level and the BMI variable between stage 2 of endometriosis and non-endometriosis as well as the combination of stadium/grade 3, 4 and non-endometriosis. Endometriosis stadium 2 had an insignificant value of NO and BMI. For the value of endometriosis of combined grade 3 and 4, the NO level had OR value=1.70 (CI 95%=1.04-2.77 with p=0.03), whereas BMI had OR value=13.63 (CI 95%=1.35-137.13 with p=0.03). Statistically NO and BMI value was significant.

With the cut off point (CoV) 7.1 of NO level, the discrimination value of AUC (area under the curve) was 17.8%. So, it can deduce that NO level as an indicator of endometriosis with 7.1 (Figure 1), and it may detect as many as > 77.8% of individuals with endometriosis. The AUC value= >77.8% is assumed to have a modest force as an instrument of filtering test.

Table 4. Multiple linear regression test between endometriosis, types of endometriosis, grades of endometriosis, dysmenorrhea, history of endometriosis in the family and nitric oxide

Variable	Model 1 *P* Coeff CI 95 %	Model 2 *P* Coeff CI 95 %	Model3 *P* Coeff CI 95 %
Endometriosis			
Endometriosis	0.01* 1.98 (0.69-3.26)	-	-
Non-endometriosis	-	-	-
Types of Endometriosis			
Chocolate (-)	-	0.02* 2.02 (0.30-3.74)	-
Chocolate (+)	-	0.00* 1.95 (0.55-3.36)	-
Non-endometriosis			
Grades of Endometriosis			
2	-	-	0.01* 2.69 (0.65-4.73)
3	-	-	0.02* 1.39 (0.17-2.62)
4	-	-	0.01* 1.46 (0.31-2.62)
Non-endometriosis			
Dysmenorrhea			
Yes	-	-	0.08 1.47 (-0.21-3.17)
No	-	-	-
History of Endometriosis			
Yes	0.63 0.48 (-1.52-2.49)	0.47 0.72 (-1.31-2.75)	0.70 0.49 (-2.06-3.05)
No			
Interaction			
Endometriosis X history of endometriosis	-	-	-0.01* 0.99 (-4.65-4.64)
Chocolate (-) X history of endometriosis	-	-	-
R2	0.29	0.30	0.29
N	54	54	54

*Significant *P*=<0.05

Table 5. Logistic regression of the NO relation with considering BMI between grade 2 and non-endometriosis between grade 3, 4

Stage/*Grade*	Variable	OR	CI 95%	*P*
2	NO	2,31	0,90-5,91	0,07
	IMT			
	>=23	2,61	0,09-71,95	0,56
	<23 (Ref)	-	-	-
3 4	NO	1,70	1,04-2,77	0,03*
	IMT			
	>=23	13,63	1,35-137,13	0,03*
	<23 (Ref)	-	-	-
R2				0,36
Deviance				60,05

Ref=non-endometriosis *Significant *P*=<0.05

Endometriosis patient experience a change in the situation of follicular fluid which will cause abnormalities of cycles. This change causes an increase in several macrophages and immunological change in the peritoneal fluid and serum of women with endome-triosis, which has an important role in the pathological change and is associated with infertility in endometriosis patient.[2]

The increased number and activity of macrophages in endometriosis patients are concurrent with a release of excessive cytokine and the immune mediator such as NO (one type of ROS). A low level of NO is critical in the function of ovary and implantation. Previous research showed that increased No and NOS found in endometriosis patients. A high level of NO in the peritoneum fluid has been reported to decrease infertility by obstructing the function of the oviduct and the mortality of sperms. Besides, NO also shows a toxic effect on the embryo and blocks implantation. However, the mechanism of how it works is not clear.

The NO level in endometriosis is higher than that in non-endometriosis and has a significant relationship with all the variables (age, parity, dysmenorrhea, dyspareunia, types of endometriosis, and grade of endometriosis) this accords with the results of research and increased NO level in endometriosis patients. A low level of NO is critical in the function of the ovary and implantation and causes relaxation of the oviduct[9] Therefore, a high level of NO has been reported to have an effect of decreasing infertility. A high expression of NO in the peritoneum of sperms and is toxic to the embryo thereby obstructing implantation NO is a free radical with a disruptive effect and is an important bio regulator of apoptosis. NO radical is a local factor that is involved in the folliculogenesis and steroidogenesis of the ovary.[10] The folliculogenesis of the ovary involves not only gonadotropin and steroid but also the local autocrine and parathyrin eNOS as the producer of NO exists in the human corpus luteum, and the eNOS expression is reported to exist in the middle phase and initial phase of the luteal of the menstrual cycle. NO in folliculogenesis and steroidogenesis exist in the corpus luteum and have a share in luteolysis which mediated by an increase in prostaglandin and apoptosis. The NO expression in the follicular fluid linked to infertility in the patient with endometriosis. NO in the peritoneum fluid in endometriosis patients has the effect of decreasing infertility in women.[11] This study accords with the result of this research that the NO level in the follicular fluid is higher than normal. NO has an imperfect relation with folliculogenesis because of the effect of apoptosis in the oocyte. The increase in NO level with implies fertility. The results of multiple linear regression lists show that the NO level growth in endometriosis taking into account the history of endometriosis in the family, types of endometriosis of chocolate cyst (+), and chocolate cyst (-). Chocolate cyst (+) has a p value=0.00, whereas chocolate cyst (-) has a p value=0.02. The NO values in all the grades of endometriosis, taking into account dysmenorrhea and history of endometriosis, have a significant difference (grade 2 p=0.01; grade 3 p=0.02; and grade 4 p=0.01).

The results of multinomial regression test show that the NO level increases in endometriosis by relating dysmenorrhea, dyspareunia, age, both in grade 2 and combination of grade 3 and 4. If the study analyzed by distinguishing chocolate cyst (-) and chocolate cyst (+), there will be a significant difference when controlled with dysmenorrhea and dyspareunia. When managed with age, the significant NO level is found only in chocolate-cyst (-).

In this research, the median of NO level was 8.32 nmol/L, which is far below the result of the investigation in Florida, USA with an average of 12 nmol/L[12] Likewise the result of research in Jerusalem, Israel, which found a median of 15 nmol/L.[13] This research shows a positive correlation between IL-1β and NO.[14] This result is congruent with the opinion that IL-1B has a positive relationship with NO among patients with non-endometriosis. NO is thought to be a variable that affects the number of follicles that develop significantly.[7]

CONCLUSION

The NO level in endometriosis is higher than that in non-endometriosis. This research also yields a new finding, i.e., the marker of laboratory examination of NO (cut off point > 7.1) as a support for the diagnosis of endometriosis. The distribution of the polymorphism of allele GG is greater than non-endometriosis. The existence of polymorphism GG causes a higher eNOS increasing NO level causing a decrease in fertility which derived from the degenerative oocyte.

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